BCL-xL/xS PHOSPHORYLATION REGULATES THE SENSITIVITY OF PC12 CELLS TO APOPTOSIS

A Thesis Submitted to the
College of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for a Master's Degree
in Biological Psychiatry
Department of Psychiatry
University of Saskatchewan
Saskatoon

By
Ji Qi

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Master's degree from the University of Saskatchewan, I agree that the Libraries of this university make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by Dr. Darrell D. Mousseau, who supervised my thesis work or, in his absence, by the head of Department of Psychiatry at the University of Saskatchewan. It is understood that any copying, publication, or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me, to the Department of Psychiatry and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or in part should be addressed to:

Dr. Darrell D. Mousseau and/or

Head of the Department of Psychiatry

University of Saskatchewan

Saskatoon, Saskatchewan

Canada, S7N 5E5

ABSTRACT

The Bcl-2 family of proteins contains both anti-apoptotic (*e.g.*Bcl-2, Bcl-xL) and pro-apoptotic (*e.g.*Bad, Bcl-xS) proteins. Bcl-xL and Bcl-xS are splice variants, but have different functions during apoptosis. The pro-survival kinase Akt can phosphorylate certain Bcl-2-related proteins, specifically on serine residues, to regulate their function and localization. This is an extension of the work from our laboratory's finding that haloperidol promotes PC12 cell death by inducing Bcl-xS which then translocates from cytosol to mitochondria and facilitates the release of cytochrome *c*. The toxicity induced by Bcl-xS is reversed by expression of constitutively active Akt. I hypothesized that Akt-mediated post-translational modification may be important for regulating the function of Bcl-xS as well as Bcl-xL.

Three specific serine residues were ultimately chosen for the characterization of Bcl-xS/xL function: Serine62 (homologous to Serine70, a major site for phosphoregulation, in Bcl-2), Serine106 (putative Akt phosphorylation motif), and Serine165 in Bcl-xS (and the corresponding Serine228 in Bcl-xL) (immediately upstream of hydrophobic tail). The individual substitutions of all three Serines with Alanines (Ser/Ala; S/A: which precludes phosphorylation at that site) in Bcl-xS did not affect the expression of the protein, but they did induce varying degrees of cytotoxicity in both PC12 and HEK cultures. I focused on the Ser106 substitution mutant given my hypothesis that Akt targeted this site. Overexpression of Bcl-xS(S106A) was toxic in both PC12 and HEK cultures, as expected, and this coincided with the appearance of the Bcl-xS(S106A) protein in the mitochondrial fraction. The release of cytochrome *c* from PC12 cell mitochondria coincided with the co-immunoprecipitation of the Bcl-xS protein with VDAC (voltage-dependent anion channel), a channel-forming protein

that is known to mediate cytochrome *c* release, and with the initiation of caspase-dependent events. This was not the case in HEK cells, where the mitochondrial VDAC seemed to be diminished and the toxicity was cytochrome *c*-independent as well as caspase-independent. In addition, I was able to demonstrate that Bcl-xS(S106A) did not co-immunoprecipitate with Akt, supporting Ser106 as a potential target for the Akt protein. I then studied the effects of the homologous substitutions in Bcl-xL on cell function. I chose to use treatment with the potent inducer of apoptosis, staurosporine, as a model of cytotoxicity. Again, substituted proteins exerted toxicity, but they did not potentiate the effects of staurosporine, at least not on MTT conversion. I did notice, however, that there was a clear morphological change with certain concentrations of staurosporine, and subsequently demonstrated that the Bcl-xL(S106A) protein potentiated PC12 cell differentiation induced by staurosporine. The Bcl-xL(S106A) protein also co-immunoprecipitated better with Akt, which was unexpected given my results with the Bcl-xS(S106A) protein described above. Perhaps the extra amino acids in Bcl-xL account for this.

It is clear that the phosphorylation of Bcl-xS and Bcl-xL proteins is an important means of regulating their function and localization within the cell. These data support the Ser106 residues in both Bcl-xS and Bcl-xL as novel targets for the pro-survival Akt kinase, and indicate a role for this/these residue(s) in cellular functions as diverse as apoptosis and differentiation.

ACKNOWLEDGMENTS

I would like to extend my gratitude to my supervisor, Dr. Darrell D. Mousseau, for giving me the opportunity to pursue my Master's degree studies in Canada, and for all of his kindly instruction and supporting me to develop my skills and work independently. It is my pleasure to have gained such a valuable scientific experience and wisdom from Dr. Darrell D. Mousseau.

I would like to express my appreciation to the members of my advisory committee, Dr. XinMin Li and Dr. Ron Geyer, for their guidance and support during my graduate studies at the University of Saskatchewan. I would also like to thank Dr. Vikram Misra, who acted as the external examiner for my thesis defense. His questions, suggestions and comments were very helpful.

I would also like to acknowledge the financial support I have received from the Department of Psychiatry (an Aruna and Kripa Thakur Award & a Laura E. Chapman Award) and from the College of Graduate Studies and Research (a Dean's Scholarship as well as a Master's Scholarship) at the University of Saskatchewan.

I would also like to thank all the members of the Cell Signalling Laboratory, my colleagues in the Neuropsychiatry Research Unit, and all of my friends in Saskatoon. It is a great pleasure for me to have met all of you and to have had an opportunity to work with you.

Lastly, I would like to extend my gratitude to my parents, YongChang Qi and Jie Gao, for their unconditional love and support throughout my life.

TABLE OF CONTENTS

PERMISSION TO USE		**i**
ABSTRACT		**ii**
ACKNOWLEDGMENTS		**iv**
TABLE OF CONTENTS		**v**
LIST OF FIGURES		**vii**
LIST OF ABBREVIATIONS		**ix**
1	**INTRODUCTION**	1
1.1	Apoptosis	1
1.1.1	The extrinsic apoptotic pathway	3
1.1.2	The intrinsic apoptotic pathway	3
1.2	The Bcl-2 family of anti-and pro-apoptotic proteins	4
1.2.1	Anti-apoptotic and pro-apoptotic Bcl-2-related proteins	5
1.2.2	The BH domain of Bcl-2 family-related proteins	5
1.2.3	Examples of Bcl-2-related proteins	7
1.2.4	Phosphorylation on Bcl-2-related proteins	8
1.2.5	Bcl-xS and Bcl-xL	10
1.3	The voltage-dependent anion channel (VDAC)	12
1.4	The PI3K/Akt pathway	13
1.5	Chemical reagents that lead to apoptosis	14
1.6	Pheochromocytoma PC12 cells	15
1.7	Summary of the thesis project	15
2	**MATERIALS AND METHODS**	17
2.1	Materials	17
2.1.1	Cell lines	17
2.1.2	Vectors	17
2.1.3	Plasmids	18
2.1.4	Competent cells	18

2.1.5 Antibodies 18
2.2 Methods 18
2.2.1 Cell culture 18
2.2.2 Subcloning of the target gene into a mammalian expression vector 19
2.2.3 Transformation of bacterial cultures 22
2.2.4 Polymerase chain reaction (PCR) 22
2.2.5 Mutagenesis of specific Serine residues 23
2.2.6 Mini-prep 26
2.2.7 Maxi-prep 27
2.2.8 Transfection of mammalian cultures 29
2.2.9 Western blot analysis 30
2.2.10 Immunoprecipitation of target protein 32
2.2.11 Immunofluorescence and double-staining 34
2.2.12 MTT conversion assay 35
2.2.13 Statistics 36

3 RESULTS 37
3.1 Specific Serine residues in pro-apoptotic Bcl-xS dictate its toxicity 37
3.2 Subcloning of Bcl-xL WT and substitution mutants 41
3.3 Specific Serine residues in anti-apoptotic Bcl-xL dictate its function 49
3.4 Staurosporine-induced toxicity is Akt-and caspase-dependent in primary neuronal cultures 49
3.5 Staurosporine-induced toxicity in PC12 cells presents with indicators of apoptosis 52

4 DISCUSSION 63

5 REFERENCES 67

6 APPENDIX 74
License agreement/permission for use of figures

LIST OF FIGURES

Figure 1.1: The extrinsic and intrinsic apoptotic signaling pathways 2

Figure 1.2: BH domains in Bcl-2 family proteins 6

Figure 1.3: Amino acid sequences of Bcl-xL and Bcl-xS 11

Figure 3.1: Overexpression of Bcl-xS Ser-to-Ala variants affects PC12 cell viability 38

Figure 3.2: Overexpression of Bcl-xS Ser-to-Ala variants affects HEK cell viability 39

Figure 3.3: Bcl-xS(S106A) exerts different effects on mitochondrial function in PC12 and HEK cells 42

Figure 3.4: The Bcl-xS(S106A) substitution influences the interaction with VDAC and with Akt 43

Figure 3.5: Plasmid map of pCMV/FLAG3-Bcl-xS and pCMV/FLAG3-Bcl-xL 44

Figure 3.6: Confirmation of the Bcl-xL cDNA insert in pCMV/FLAG3 using restriction analysis 45

Figure 3.7: The chromatogram covering the Bcl-xL WT cDNA sequence 46

Figure 3.8: Sequencing confirms the Bcl-xL S62A, S106A and S228A mutations 48

Figure 3.9: Dephosphorylation of selected serine residues of anti-apoptotic Bcl-xL reveals a cytotoxic profile 50

Figure 3.10: Staurosporine-induced primary cell toxicity is Akt- and caspase-dependent 51

Figure 3.11: Staurosporine-induced PC12 cell apoptosis 53

Figure 3.12: Overexpression of Bcl-xL WT and S106A proteins does not alter the staurosporine-induced loss of MTT conversion in PC12 cells 54

Figure 3.13: Bcl-xL(WT & S106A) interacts with pro-apoptotic Bax in staurosporine-treated PC12 cells 55

Figure 3.14: Mutation of Bcl-xL at Ser106 (a potential Akt target) induces more of an interaction with Akt in staurosporine-treated PC12 cells 58

Figure 3.15: Bcl-xL(S106A) promotes ST-induced differentiation of PC12 cells 60

Figure 3.16: Staurosporine induces MAP-2 cleavage in Bcl-xL(S106A)-expressing PC12 cells 61

Figure 3.17: Neither Bcl-xL wildtype nor Bcl-xL(S106A) interacts with VDAC in PC12 cells 62

LIST OF ABBREVIATIONS

A/Ala	Alanine
Amp	Ampicillin
ANT	Adenine nucleotide translocator
APAF 1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
Caspase	Aspartic acid specific cysteine protease
CMV	Cytomegalovirus
DAPI	4,6-diamidino-2-phenylindole
DISC	Death inducing signal complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescent luminal
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinase
FADD	Fas associated death domain
FasL	Fas ligand
HAL	Haloperidol
HBSS	Hank’s Balanced Salt Solution
HEK	Human embryonic kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
IB	Immunoblot
IP	Immunoprecipitation
LB	Loading buffer
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MEK	Mitogen and extracellular signal-activated protein kinase kinase

MOMP	Mitochondrial outer membrane permeabilization
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP ribose) polymerase
PBS	Phosphate-buffered saline
PC 12	Pheochromocytoma
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI3K	Phosphatidylinositol 3'-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
S/Ser	Serine
SAPK	Stress activated protein kinase
SDS	Sodium dodecylsulfate
ST	Staurosporine
T/Thr	Threonine
TB	Terrific Broth
TBE	Tris-borate-EDTA
TBST	Tris-buffered saline with Tween 20
TCL	Total cell lysate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TM	Transmembrane
TNFR	Tumor necrosis factor receptor
VDAC	Voltage-dependent anion channel
WT	Wild type

1. Introduction

1.1 Apoptosis

Apoptosis is a mechanism of cell 'suicide' designed to maintain homeostasis and often is associated with programmed cell death. The actual mechanism involves a series of biochemical events leading to a characteristic change in cell morphology that includes chromatin aggregation, nuclear and cytoplasmic condensation, nuclear fragmentation, release of cytochrome *c* from the mitochondria to the cytosol and, ultimately, the shrinkage of the cell and its breakup into pieces called apoptotic bodies. There are two main apoptotic pathways, termed the intrinsic and extrinsic pathways (Fig. 1.1) [1-4]. Occasionally, these two pathways cross-talk and form an amplified feedback loop [1, 4].

Aspartic acid-specific cysteine proteases (also known as cysteine aspartate-specific proteases or *caspases*) are a family of enzymes that are essential for intrinsic as well as extrinsic apoptotic pathways. There are two types of caspases that are generally characterized according to differences in structure and function. 'Initiator caspases' include caspase-2, -8, -9, and -10, and activate apoptotic processes by cleaving (and, thus, activating) the pro-forms (inactive) of effector caspases. 'Effector caspases' (also called 'executioner caspases') include caspase-3, -6, and -7, all of which cleave other proteins that trigger the irreversible (hence 'executioner') aspects of the apoptotic process [1, 2]. Caspase-8 is a mediator of the extrinsic pathway whereas caspase-9 mediates the intrinsic pathway.

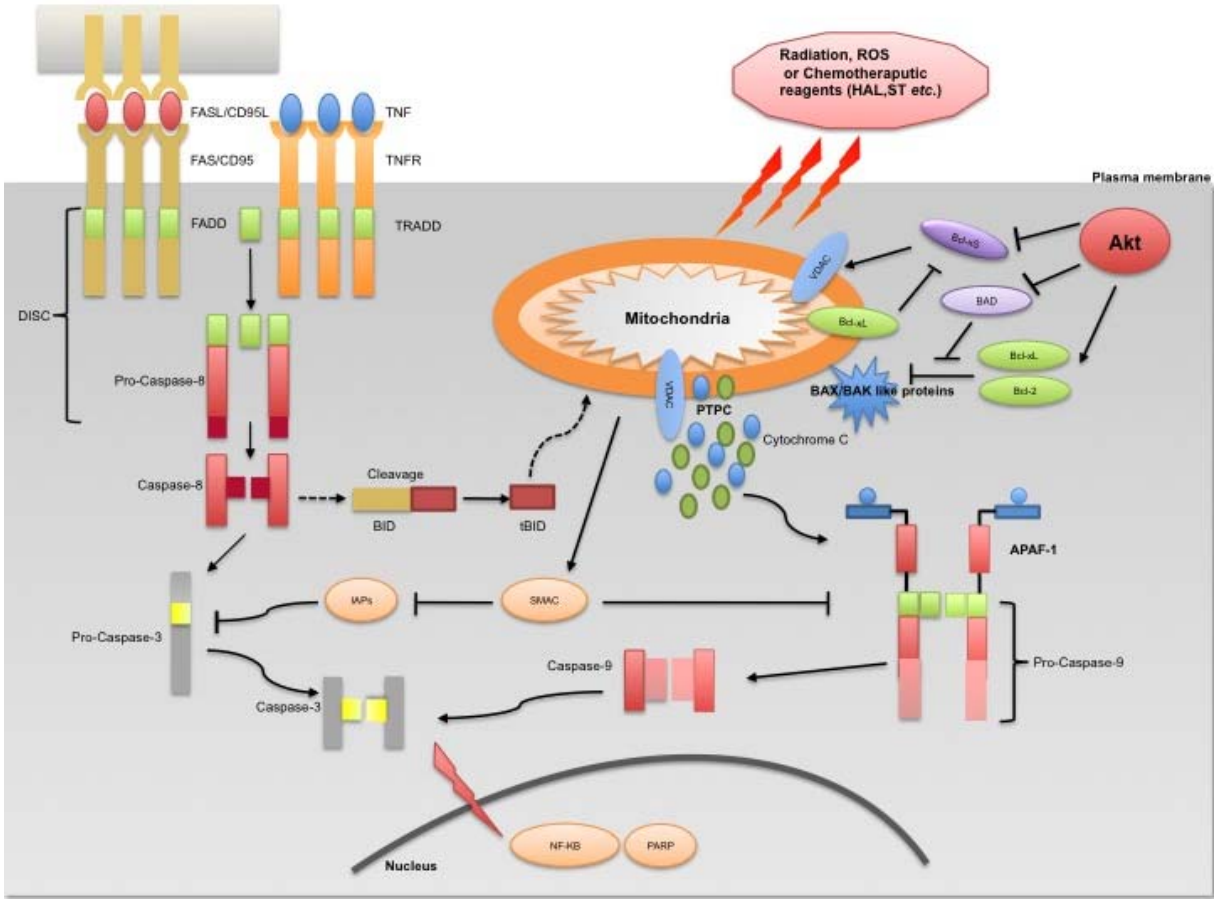


Figure 1.1: The extrinsic and intrinsic apoptotic signaling pathways. The two pathways mediating cell apoptosis are called the extrinsic apoptotic pathway, also known as death-receptor mediated pathway (pathway on the left), and the intrinsic apoptotic pathway, also known as mitochondria pathway (pathway on the right). In Caspase-dependent apoptosis, caspase-8 is an example of a regulator of the extrinsic apoptotic pathway whereas caspase-9 is an example of a regulator of the intrinsic apoptotic pathway. Those two pathways can cross-talk to amplify the apoptotic signal. (Adapted from Ref [1])

1.1.1 The extrinsic apoptotic pathway

The extrinsic apoptotic pathway is also known as the death-receptor regulated pathway. When a death ligand, such as FasL (Fas ligand) or TNF (tumor necrosis factor), binds to its corresponding receptor (*e.g.* Fas or TNF receptor, respectively), it recruits the pro-form of initiator caspases that are cleaved and then dimerized. Once activated, they, in turn, activate the effector caspase (Fig. 1.1). The activated effector caspase targets several substrates such as the transcription factor (NF-kB) or the enzyme poly(ADP-ribose) polymerase (PARP: required for efficient DNA repair of single-strand breaks) [5], either of which promotes an apoptotic phenotype.

Fas is a type I transmembrane receptor protein and FasL is a type II transmembrane protein. Fas can mediate apoptosis by ligation with FasL; a Fas trimer binds to the Fas-associated death domain (FADD) protein which acts as a bridge between death receptors and caspases. Oligomerization of FADD binds pro-caspase-8 and forms the death-inducing-signal-complex (DISC). Caspase-8 then dimerizes and dissociates from DISC, and then is freed to activate the effector caspase-3 in the cytosol [4, 6].

TNF binds TNFR to initiate another cascade. For example, TNF-α binds TNFR I, and TNFR I is trimerized and recruits an adaptor protein called TNFR-associated death domain (TRADD). TRADD can then bind with FADD to form a dimer. This then activates, as described above, pro-caspase-8 and initiates apoptosis [6].

1.1.2 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is also known as the mitochondrial apoptotic pathway given the crucial role of mitochondrial outer membrane permeabilization (MOMP) in

this pathway [7]. The regulation of MOMP depends, in large part, on modulation by the function of the voltage-dependent anion channel (VDAC). When a cell is affected by stressors such as radiation, reactive oxygen species (ROS) or chemotherapeutic reagents, the mitochondria can respond, because of a change in VDAC function and MOMP, by releasing cytochrome *c* to the cytosol. Cytosolic cytochrome *c* binds the apoptotic-protease-activating-factor 1 (APAF1) and pro-caspase-9 to form the apoptosome. The apoptosome activates the effector caspase-3 and eventually leads to apoptosis [2-4]. In addition, a second mitochondrial-derived activator of caspase can be released from mitochondria and blocks the inhibitor-of-apoptotic proteins and exacerbates the apoptotic signal.

These apoptotic cascades are tightly regulated, for obvious reasons. Proteins such as those belonging to the Bcl-2 family of proteins can influence a cell's response to a stressor by either promoting or inhibiting the apoptotic process. This can be done, for example, by regulating MOMP or by regulating cytochrome *c* function [8].

1.2 The Bcl-2 family of anti- and pro-apoptotic proteins

The B-cell lymphoma 2 (Bcl-2) family of proteins comprises both anti-apoptotic (*e.g.* Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic (*e.g.* Bad, Bax, Bcl-xS) members, and plays a prominent role in regulating apoptosis. Bcl-2 family members are able to promote or inhibit apoptosis directly by regulating MOMP or indirectly by regulating the function(s) of other protein(s). Bcl-2-related proteins contain Bcl-2 homology (BH) domains that allow them to form homo- or heterodimers that ultimately define their anti- or pro-apoptotic functions. Recent studies reveal that the phosphorylation of Bcl-2 family proteins is important in regulating their function during apoptosis. The following is a brief discussion of Bcl-2-related

proteins as well as the various mechanisms known to regulate their influence on cell viability.

1.2.1 Anti-apoptotic and pro-apoptotic Bcl-2-related proteins

Bcl-2, Bcl-xL, Bcl-W, Bcl-2A1, MCL1, and BOO are anti-apoptotic members of Bcl-2 family; Bax, BOK, Bcl-xS, Bak, Bad, BIK, BID, HRK and BIM are all examples of pro-apoptotic members of the same family [3]. Bcl-2 was discovered in the 1980s as a proto-oncogene. Overexpression of Bcl-2 could protect against neuronal cell death by arresting cell cycle at the G1 stage [9, 10]. The various Bcl-2 homology (BH) domains in this protein allow for the formation of heterodimers with pro-apoptotic Bcl-2-related proteins (and thereby sequestering them and arresting apoptosis).

1.2.2 The BH domain of Bcl-2 family-related proteins

The Bcl-2 family of proteins is characterized by the presence of specific regions of homology, the aforementioned Bcl-2 homology (BH1, BH2, BH3 and BH4) domains (Fig. 1.2). BH domains are critical to the function of Bcl-2 family proteins and alter their impact on cell survival and their ability to interact with other family members and regulatory proteins [3, 11]. BH domains allow Bcl-2 family members to interact and form homo- or heterodimers.

Bcl-2 family proteins contain both anti- and pro- apoptotic protein members. The anti-apoptotic members all have four BH domains (the exception being MCL1, which only has BH1 and BH3 domains). The flexible loop structure between BH3 and BH4 domains in Bcl-2 and Bcl-xL is believed to be a regulatory region for their function [12]. The pro-apoptotic protein members can be divided into two subgroups: (i) the Bax/Bak-like proteins and (ii) the BH3-only proteins. The Bax/Bak-like proteins contain at least two BH domains,

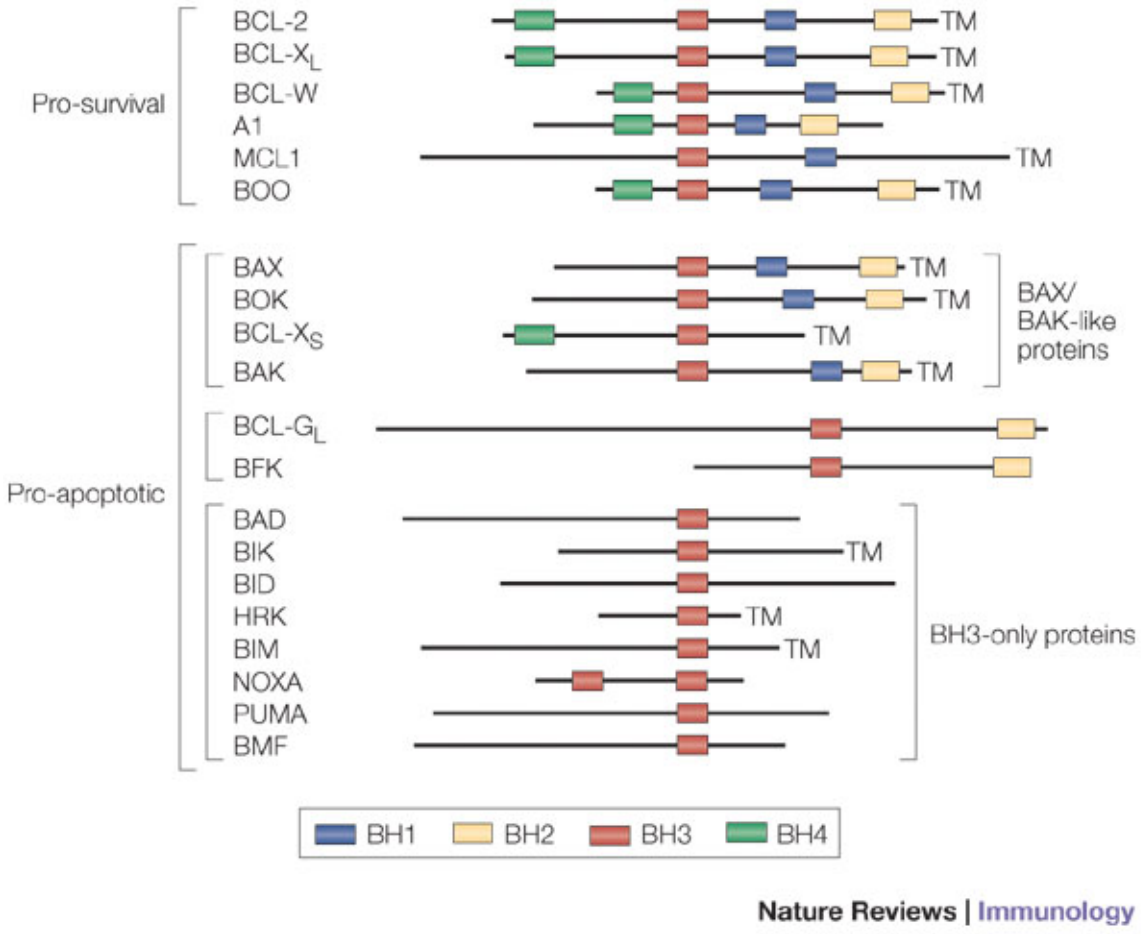


Figure 1.2: BH domains in Bcl-2 family proteins. Bcl-2 family contains both anti- and pro-apoptotic protein members. All anti-apoptotic members contain the four BH domains, except MCL1 which only has BH1 and BH3 domains. The pro-apoptotic members can be divided into two subgroups: (i) the Bax/Bak-like proteins which contain at least two BH domains, and (ii) the BH3-only proteins. TM: transmembrane domain, allows Bcl-2 family members to target membranes. (Used with permission; Ref [3])

whereas the BH3-only protein members, as their name implies, only contain a BH3 domain (Fig. 1.2). The BH3 domain has high affinity for, and inactivates, anti-apoptotic proteins [3], yet the BH3-only protein, BID, does not interact with anti-apoptotic Bcl-2 members, but is actually cleaved by caspase-8 and mediates Fas/FasL-mediated apoptosis [13, 14]. The transmembrane domain (TM) allows Bcl-2 family member to target intracellular membranes, such as the outer membrane of the mitochondria.

1.2.3 Examples of Bcl-2-related proteins

MCL1 (myeloid-cell leukemia sequence 1) promotes cell viability and interrupts apoptotic signal by binding with pro-apoptotic members (*e.g.* BIM). MCL1 has a very short half-life [15] and its function is regulated at the post-translational level. MCL1 is a target for proteasome degradation and the process is phosphorylation-dependent [16, 17]. BOO (Bcl-2 homologue of ovary, also known as DIVA) is a newly discovered regulator of apoptosis that contains four BH domains unlike MCL1, which only has two. BOO inhibits the binding of Bcl-xL with APAF1 [18], but there is lack of evidence in support of its anti-apoptotic activity.

Two classes of proteins comprise the pro-apoptotic members of the Bcl-2 family. One is Bax/Bak-like protein which includes Bax, BOK, Bcl-xS and Bak, whereas the other includes the BH3-only proteins Bad, BIK, BID, HRK and BIM [3]. The following is a cursory description of each protein.

Although Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist killer) are structurally similar to the anti-apoptotic Bcl-xL, and are normally sequestered and inactivated by heterodimerization with Bcl-xL within the cytosol, they are actually pro-apoptotic and function by translocating from the cytosol to the mitochondria where they regulate the

opening of the permeability transition pore complex [19]. This allows for cytochrome *c* release to the cytosol and caspase activation. The effects of BOK (Bcl-2 related ovarian killer) are not antagonized by anti-apoptotic Bcl-2-related proteins such as Bcl-2 or Bcl-xL and appear to depend on its ability to localize to the nucleus (by virtue of a Leucine-rich sequence within its BH3 domain) [20].

BH3-only proteins include Bad (Bcl-2 antagonist of cell death), which transduces the death signal to the mitochondria by interfering with the association between Bcl-xL and Bax. This not only allows Bax to translocate to the mitochondria and affect VDAC function, but it also diminishes the ability of Bcl-xL to bind with APAF1 or other pro-apoptotic proteins [21]. BIK (Bcl-2 interacting killer) can bind with various anti-apoptotic proteins through its BH3 domain, but the heterodimer alone is not sufficient to induce apoptosis; it is believed that phosphorylation of BIK may promote its toxicity [22]. HRK (hara-kiri, also known as DP5) is regulated by the transcription factor pRB [23] and mediates apoptosis related with growth factor withdrawal [24]. BIM (Bcl-2 interacting mediator of cell death) promotes apoptosis by binding with LC8 cytosol dynein light chain and Bcl-2. This releases, and activates, Bax [25, 26]. BID (BH3 interacting domain death agonist) is very important in Fas-mediated apoptosis since BID is cleaved by caspase-8 and the truncated BID (tBID) translocates from cytosol to mitochondria and triggers the release of cytochrome *c* (Fig.1.1a).

1.2.4 Phosphorylation on Bcl-2-related proteins

It is known that structural features such as BH domains dictate the inherent function of Bcl-2-related proteins. However, clinical data based on small cell lung carcinoma [27] or breast cancer cells [28] have revealed that increased expression of Bcl-2 is itself not sufficient

to suppress drug-induced apoptosis and chemoresistance. These findings suggest that additional factors, such as post-translational modification, *e.g.* phosphorylation, could also regulate its function [29, 30]. Phosphorylation of some Bcl-2 family members, especially on serine residues, can influence their anti- or pro-apoptotic function.

For example, Bcl-2 can be phosphorylated at the flexible loop domain on Ser70 by interleukin-3 or protein kinase C to support prolonged cell survival [12, 31]. It can also be phosphorylated on Thr56 site by JNK (a subgroup of mitogen activated kinases) during mitosis [32]. JNK can also phosphorylate Bcl-2 on additional Serines [33, 34] and either activate or inhibit its anti-apoptotic function.

Bcl-xL is phosphorylated at the activation loop on Ser62 (homologous to Ser70 on Bcl-2, above) by JNK, which not only modulates the Bax/Bcl-xL interactions [35], but also inhibits Bcl-xL function [36]. Phosphorylation on Thr47 and Thr115 by SAPK/JNK (stress activated protein kinase) also promotes apoptosis, and, not surprisingly, overexpression of Bcl-xL bearing substitution of these Serines with Alanines (thus precluding phosphorylation on these sites) promotes cell survival during under stress [37].

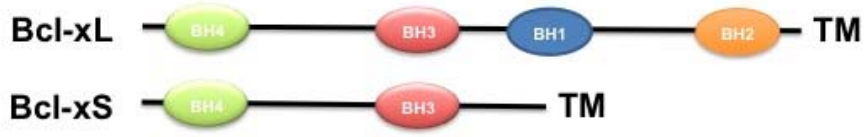
Phosphorylation on MCL1Ser64 by CDK during G_2/M phase enhances the binding of MCL1 with BIM and Bak to inhibit apoptosis [15], and phosphorylation on Thr163 can prolong it's half-life [16]. BIM is phosphorylated on Ser87 by Akt, which may diminish its apoptotic function by increasing its binding with the cytoplasmic 14-3-3 proteins (which would sequester it) [26]. Bax is also phosphorylated by Akt on Ser184, which retains Bax in the cytosol and inhibits its ability to translocate to the mitochondria and initiate the mitochondrial apoptotic pathway [38]. In contrast, phosphorylation of Bax on Ser163 by GSK-3β promotes its translocation to the mitochondria and the induction of apoptosis [39].

BID is phosphorylated by casein kinase I and II on Ser61, Ser64 and Ser78 residues, all of which, when phosphorylated, attenuate caspase-8-mediated BID cleavage, thereby blocking Fas-mediated apoptosis [13, 14]. Bad displays pro-apoptotic activity when heterodimerized with Bcl-2 or Bcl-xL, and can be phosphorylated at multiple sites, *e.g.* on Ser112 by ERK and on Ser136 by Akt [40], which promotes its binding with the cytoplasmic 14-3-3 protein. The binding of Bad with 14-3-3 impedes its ability to disrupt the Bax:Bcl-2 (or Bax:Bcl-xL) heterodimers and promotes cell survival. Phosphorylation of Bad on Ser136 also permits subsequent phosphorylation on Ser155 by protein kinase A [41]. Finally, phosphorylation of Bad on Ser170 [42] is also important for promoting cell survival.

1.2.5 Bcl-xS and Bcl-xL

Pro-apoptotic Bcl-xS and anti-apoptotic Bcl-xL are derived from the same gene, but are splice variants: x<u>S</u> represents the <u>s</u>hort form of the gene/protein, whereas x<u>L</u> represents the <u>l</u>ong form of the gene/protein (Fig.1.3). The Bcl-xL gene encodes 233 amino acids and contains all four BH domains, whereas the shorter Bcl-xS gene encodes 170 amino acids and only contains the BH3 and BH4 domains near its transmembrane domain (TM).

The pro-apoptotic protein Bcl-xS is normally expressed at very low levels and is primarily cytosolic. However, when it is overexpressed, Bcl-xS causes caspase activation and apoptosis [43] as a result of its ability to translocate to the mitochondria where it interacts with VDAC and facilitates cytochrome *c* release to the cytosol [19, 44-46]. Its apoptotic function is inhibited by co-overexpression of Bcl-xL.




```
Bcl-XL      1         MSQSNRELVVDFLSYKLSQKGYSWSQFSDVEENRTEAPEETEAERETPSAINGNPSWH
Bcl-XS      1         MSQSNRELVVDFLSYKLSQKGYSWSQFSDVEENRTEAPEETEAERETPSAINGNPSWH

Bcl-XL      59        LADSPAVNGATGHSSSLDAREVIPMAAVKQALREAGDEFELRYRRAFSDLTSQLHITP
Bcl-XS      59        LADSPAVNGATGHSSSLDAREVIPMAAVKQALREAGDEFELRYRRAFSDLTSQLHITP

Bcl-XL      117       GTAYQSFEQVVNELFRDGVNWGRIVAFFSFGGALCVESVDKEMQVLVSRIASWMATYL
Bcl-XS      117       GTAYQSFEQ.....................................................

Bcl-XL      175       NDHLEPWIQENGGWDTFVDLYGNNAAAESRKGQERFNRWFLTGMTVAGVVLLGSLFSR
Bcl-XS      175       .....DTFVDLYGNNAAAESRKGQERFNRWFLTGMTVAGVVLLGSLFSR

Bcl-XL      233       K
Bcl-XS      233       K
```

Figure 1.3: Amino acid sequences of Bcl-xL and Bcl-xS. (top) Schematic of the presence and distribution of BH domains in Bcl-xL and Bcl-xS. (bottom) The deduced amino acid sequences for Bcl-xL and Bcl-xS are aligned and show great sequence identity, which is not unexpected as they are splice variants. Specific Serine residues (shown in red) were ultimately chosen for the characterization of Bcl-xS/xL function. Ser62 (inactivation mutant, based on homology to Ser70 in Bcl-2), Ser106 (putative Akt phosphorylation motif), and Ser165 in Bcl-xS or Ser228 in Bcl-xL (immediately upstream of hydrophobic tail and homologous to Ser184 in Bax); the dotted line represents the amino acids missing due to an exon deletion from the Bcl-xL gene, making the overall gene/protein shorter, e.g. Bcl-xS ("S" for short). BH1, BH2, BH3 and BH4: Bcl-2 homology domains.

The anti-apoptotic protein Bcl-xL normally localizes to the outer membrane of mitochondria where it regulates VDAC function and blocks caspase activation and cell death .

1.3 The voltage-dependent anion channel (VDAC)

Mitochondria play a crucial role in regulating the intrinsic apoptotic pathway. In this pathway, caspase activation is closely related to MOMP [7], which depends on the function of the permeability transition pore complex [47].

The permeability transition pore complex consists of a VDAC-ANT (adenine nucleotide translocator) complex and other proteins such as peripheral benzodiazepine receptor, Hexokinase II, and cyclophilin D [47, 48]. VDAC is expressed on the outer mitochondrial membrane, where it interacts with several cytosolic proteins and mitochondrial proteins such as Bcl-2 family members which regulate (block or promote) the release of cytochrome *c* to cytosol [19, 44, 49-51]. The ANT is expressed on the inner membrane of mitochondria and allows the exchange of ADP and ATP at the inner membrane.

When a cell receives a stress signal such as increased oxidative stress or a high level of Ca^{2+}, the result can be the decrease in mitochondrial membrane potential. This causes disruption of the VDAC-ANT pore complex (which normally connects the inner and outer mitochondria membrane to allow for ATP transfer from the inside of the mitochondria to the cytosol). The disrupted pore complex now allows the "leaking" of cytochrome *c* from the intramembrane space into the cytosol where it activates the caspase cascade, eventually leading to DNA damage and apoptosis. Pro-apoptotic Bcl-2 family members, such as Bax and Bak, can facilitate the pore dysfunction by interacting with VDAC, but the action is inhibited by the anti-apoptotic proteins Bcl-2 and Bcl-xL . The BH1 and BH4 domains of Bcl-2 and

Bcl-xL play an important role in maintaining pore closure [46]. Interestingly, the function and interaction of Bcl-2-related proteins such as Bcl-xS and Bcl-xL with VDAC is, in part, regulated by Serine phosphorylation downstream of the PI3K/Akt signaling pathway [44, 52, 53].

1.4 The PI3K/Akt pathway

The phosphatidylinositol-3'-kinase (PI3K)/Akt pathway is involved in functions as diverse as cell survival, proliferation and differentiation [54, 55]. Recent studies also suggest that PI3K/Akt pathway is involved in human tumor formation and angiogenesis, making it a suitable candidate for therapeutic treatment [56, 57].

Akt, also known as protein kinase B, is a Serine/Threonine kinase and the downstream target of PI3K. It is now acknowledged that Akt is activated directly by the Serine/Threonine kinase 3'-phosphatidylinositol dependent kinase-1 (PDK1) and that Akt function is limited by the protein and lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10).

PI3K, a plasma membrane-associated kinase that often interacts with receptor Tyrosine kinases (*e.g.* growth factor receptors), contains two subunits: the p85 regulatory subunit and p110 catalytic subunit. The p85 subunit relocalizes PI3K from the cytosol to the plasma membrane. At the membrane, the p110 catalytic subunit phosphorylates phosphatidylinositol at the 3' position of the hydroxyl group and generates lipid product such as PI3,4,5P_3 and PI3,4P_2. These lipid products recruit PDK1 to the membrane where it gets phosphorylated, thus activating it. These same lipid products also recruit Akt to the membrane. The proximity to PDK1 allows the latter to phosphorylate Akt on Thr308, which facilitates

the subsequent phosphorylation of Akt on Ser473 [58, 59]. Only once both Thr308 and Ser473 of Akt are phosphorylated is Akt fully activated and able to target a pool of substrates including GSK-3β (important for cell cycle entry) [60], S6K (important for protein translation) [61, 62], and also Bax, Bad, BIM, and Bcl-xL [44, 53, 59, 63, 64].

Some psychiatric medications such as antipsychotics and antidepressants also affect PI3K/Akt function. Sertraline (antidepressant; 5-HT uptake inhibitor) kills melanoma cells by inhibiting Akt phosphorylation [65], whereas antipsychotics such as phenothiazines or haloperidol (at higher dose) inhibit the Akt pathway in human ovary cancer cells [66] and in PC12 cells [67], respectively. This ability to kill cells *via* inhibition of Akt, while not helpful in treating mental disorders, could be useful in cancer treatment, where apoptosis is desirable.

1.5 Chemical reagents that lead to apoptosis

Haloperidol (HAL) is a typical antipsychotic used in the treatment of schizophrenia, but its adverse effects, including cognitive deficit, were higher than those associated with other antipsychotic drugs [68-71]. Patients are currently prescribed other antipsychotic drugs which present less adverse effects. Due to its toxicity, HAL is not approved for behavioral treatment of the elderly, particularly those with dementia, yet HAL, because of its low cost, remains one of the most commonly prescribed antipsychotic drugs worldwide to treat patients with positive symptoms of schizophrenia [72]. Higher doses of HAL (50-100 μM) are routinely used in experimental settings because of their ability to induce cytotoxicity and, particularly, apoptosis [69, 70].

Staurosporine is a potent phospholipid/Ca^{2+}-dependent kinase inhibitor [73, 74]. It induces apoptotic cell death by activating caspase-3 in neural stem cells [75] and osteoblasts

[76], and inhibits Akt [77], thus making it a potential cancer therapeutic [74]. While staurosporine is used regularly to study cytotoxicity *in vitro* [78-81] and to study nuclear condensation (a marker for apoptosis) and cell cycle arrest at G1/S phase [82], it also induces neurite outgrowth (a sign of differentiation) in PC12 cells [83, 84].

1.6 Pheochromocytoma PC12 cells

PC12 cells are derived from a pheochromocytoma and under normal conditions they develop properties similar to immature rat adrenal chromaffin cells. When grown in the presence of nerve growth factor, PC12 cells exhibit neurite outgrowth, become electrically excitable, are more responsive to exogenous acetylcholine and synthesize more neurotransmitters, particularly noradrenaline [85, 86]. There are mainly two cascades that contribute to the apoptosis in PC12 cells. One is Bax-dependent and caspase-independent cascade, whereas the other is Bcl-xS- and caspase/mitochondrial-dependent [43, 44, 87]. Thus, PC12 cells represent a good *in vitro* model for examining the influence of the respective cascades in drug-induced apoptosis.

1.7 Summary of the thesis project

The Bcl-xL and Bcl-xS proteins are splice variants, but exert different functions during apoptosis. In previous studies from our laboratory, we found that haloperidol induces PC12 cell death by inducing (gene and protein) Bcl-xS, which translocates from the cytosol to the mitochondria and facilitates the release of cytochrome *c*. This translocation of Bcl-xS appears to be regulated by the [de]phosphorylation of specific Serine residues. Furthermore, Bcl-xS-induced toxicity is reversed by constitutively active Akt [44]. These combined data

suggest an Akt-sensitive post-translational modification of the Bcl-xS protein that determines its localization and toxicity profile. Interestingly, Bcl-xL function is also regulated, in part, by the Serine phosphorylation downstream of the PI3K/Akt survival signaling pathway [52, 53].

Based on these observations, I hypothesized that the activity of Bcl-xL in PC12 cells is regulated by the phosphorylation of specific Serine residues and that this could alter its ability to protect against specific chemical inducers of apoptosis. I chose to compare and contrast the effects of Bcl-xL to those of Bcl-xS.

I chose to target three specific Serine residues in Bcl-xL, *e.g.* Ser62, Ser106 and Ser228 (homologous to Ser165 in Bcl-xS) (shown in Fig.1.3) for mutagenesis. Therefore, these were substituted to Alanines which precludes phosphorylation on these sites. This was done so as to examine:

1. If the substituted Bcl-xS and Bcl-xL variants affect the viability of PC12 pre-neuronal cells and/or HEK293 non-neuronal cells;
2. If so, do the variants exert an effect that is different from the parental wildtype protein;
3. If the substitutions affect the proteins' ability to interact with other proteins under conditions of cell stress, *e.g.* during treatment with staurosporine;
4. Whether mutagenesis of Ser106, which resides within a putative Akt consensus motif, affects the proteins' ability to respond to the PI3K/Akt pathway.

2. Materials and Methods

2.1 Materials

All materials and reagents used in this series of experiments were obtained from commercial sources (company indicated in brackets after the reagent). The name and company address are listed in Table 2.1.

2.1.1 Cell lines

The rat pheochromocytoma (PC12, ATCC, CRL-1721), human embryonic kidney 293A cell (HEK293A, ATCC, CRL-1573) and rat primary cortical cultures were used during these experiments. PC12 cells are pre-neuronal cells and can exhibit neurite outgrowth upon differentiation. HEK293A cells are non-neuronal cells and have a high capacity for protein overexpression.

2.1.2 Vectors

The expression vector pcDNA3.1 (+) (Invitrogen) is used for high level of protein overexpression in mammalian cells. pCMV/FLAG3 was kindly provided by Dr. Deborah Anderson (Saskatchewan Cancer Center, University of Saskatchewan) and is used for overexpression and isolation of FLAG-tagged proteins. Both vectors carry the ampicillin resistance gene (Amp^+).

2.1.3 Plasmids

Bcl-xL wildtype (WT) cDNA was amplified from the pcDNA3-Bcl-xL plasmid (Richard Youle, NINDS, NIH) and subcloned into pCMV/FLAG3. pCMV/FLG3-Bcl-xL was then used to generate the S62A, S106A and S228A substitutions using the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

2.1.4 Competent cells

The DH5α *E. coli* bacterial strain was used to make competent cells (used for transforming with plasmid cDNA, see Section 2.2.3, below) and was obtained from ATCC.

2.1.5 Antibodies

The antibodies used in this thesis are indicated below in Table 2.2. Primary antibodies were diluted by using 5% BSA (EMD Chemicals Inc.) in TBS-Tween 20, incubated at 4 ºC for overnight. Secondary antibodies were diluted in 5% milk in TBS-Tween 20. All antibodies are either stored in 4ºC or -20ºC according to the manufacturer's specifications.

2.2 Methods

2.2.1 Cell culture

PC12 cells were incubated at 37ºC in Ham's F-12 Kaighn's Modification (Hyclone) nutrient medium supplemented with 15% horse serum and 2.5% fetal bovine serum in an atmosphere with an additional 5% CO_2. Culture plates and flasks for PC12 cells were pre-coated with rat-tail collagen (BD Bioscience).

HEK293A cells were incubated at 37ºC in 5% CO_2 and maintained in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum.

Rat primary cultures were prepared from time-pregnant (*i.e.* the date of conception is known) Sprague-Dawley rats [88]. E20 fetuses were anesthetized and decapitated. The brains were dissected and the cortical area was removed into Hank's Balanced Salt Solution (HBSS, GIBCO) supplemented with 15 mM HEPES and 30U/ml penicillin/streptomycin (all from GIBCO). The cortical tissue was digested at 37ºC with Trypsin-EDTA (Sigma) for 15 min. An equal volume of FBS was added to stop digestion, and the tissue was then rinsed in HBSS three times to remove any FBS. The tissue was then disrupted with several aspirations into a glass Pasteur pipette. HBSS was then added to a final volume of 5 ml. After centrifugation, the HBSS was removed and the cells were re-suspended in NeuroBasal medium with 1% N_2, 2% B27, 25 μM L-glutamate, and 20U/ml penicillin/streptomycin. Neuronal cells were plated into 96-well plates pre-coated with 25 μg/ml Poly-D-Lysine in a density of 8×10^4/well and incubated at 37ºC with 5% CO_2-humidified atmosphere. The NeuroBasal medium (without L-glutamate) was replaced after the first day and replaced again after 5 days culture.

2.2.2 Subcloning of the target gene into a mammalian expression vector

The Bcl-xL wildtype gene was amplified from the Bcl-xL/pcDNA3 plasmid. Both PCR fragment and pCMV/FLAG3 vector were restricted with *EcoR I* and *Bgl II* (Fermentas) at 37ºC for 2 hours. The purified fragments were then ligated using T4 DNA ligase (Fermentas) (16ºC overnight). After transformation, positive clones were detected by colony PCR, with PCR fragments being visualized by agarose gel electrophoresis containing 1% ethidium bromide (EB) and compared to DNA molecular weight standards (Fermentas).

Table 2.1

Company	**Address**
American Type Culture Collection	Manassas, VA, USA
BD Biosciences	Mississauga, ON, CANADA
BDH Inc	Toronto, ON, CANADA
Bio-Rad	Hercules, CA, USA
Cell Signaling Technology, Inc	Danvers, MA, USA
EMD Biosciences Inc	San Diego, CA, USAA
EMD Chemicals Inc	Gibbstown, NJ, USAA
Fermentas Inc	Burlington, ON, CANADA
GIBCO-BRL	Gaithersburg, MD, USA
Hyclone	Logan, UT, USA
ICN Biomedicals	Aurora, OH, USA
Invitrogen	Carlsbad, CA, USA
Pierce	Rockford, IL, USA
Santa Cruz Biotechnology, Inc	Santa Cruz, CA, USA
Sigma-Aldrich	St. Louis, MO, USA
Stratagene	La Jolla, CA, USA
VWR	West Chester, PA, USA

Table 2.2

Antibody	Dilution Ratio	Source	Company
primary antibody			
Phospho-Akt (Ser473)	1:750	Rabbit	Cell Signaling
Phospho-Akt (Thr308)	1:750	Rabbit	Cell Signaling
Akt	1:750	Rabbit	Cell Signaling
Bcl-xL	1:500	Mouse	Santa Cruz
Bcl-xS	1:500	Rabbit	Santa Cruz
Bcl-xL/S	1:1000	Mouse	Santa Cruz
Bax	1:750	Rabbit	Santa Cruz
Bax-NT	1:750	Rabbit	Upstate
Phospho-Bad (Ser136)	1:500	Rabbit	Cell Signaling
Bad	1:400	Rabbit	Cell Signaling
Caspase-3	1:1000	Rabbit	Santa Cruz
Caspase-9	1:500	Rabbit	Santa Cruz
MAP2	1:750	Rabbit	Santa Cruz
PARP	1:750	Rabbit	Santa Cruz
Fodrin	1:1000	Mouse	Sigma
anti-FLAG	1:1000	Mouse	Sigma
VDAC	1:750	Mouse	Calbiochem
Cytochrome *c*	1:500	Mouse	BD Bioscience
β-actin	1:3000	Mouse	Sigma
secondary antibody			
Goat Anti-Mouse IgG -HRP Conjugate	1:2000		Bio-Rad
Goat Anti-Rabbit IgG -HRP Conjugate	1:2000		Bio-Rad

2.2.3 Transformation of bacterial cultures

Transformation is a basic technique that introduces a foreign plasmid into bacteria and uses the bacteria's "machinery" to amplify the plasmid (in order to get large amounts of that plasmid). The procedure for transformation is as follows:

1. Defrost DH5α competent cells (from -70ºC stock) on ice.
2. Put 1 µl of plasmid cDNA into a 15 ml BD Falcon tube and add 50 µl of DH5α competent cells and incubate on ice for 30 min.
3. Heat shock the competent cell/ plasmid DNA mix at 42ºC (waterbath) for 45 sec.
4. Put the tube back on ice for 2 min and add 250 µl of SOC medium
5. Incubate the tube at 37ºC in an incubator/shaker for 1 hour.
6. Spread bacterial culture on DYT/Amp^+ plate; incubate at 37ºC for 16-18 hours.
7. Pick up colonies (these are ampicillin resistant and, thus, must have been transformed with the plasmid) and screen for positive clones by colony PCR.

2.2.4 Polymerase chain reaction (PCR)

The following general PCR protocol (total reaction volume 50 µl) was used to test for the presence of a cDNA insert in a particular plasmid or for colony screens (using the actual bacterial colony), which tests for a cDNA insert following a ligation/transformation experiment. Optimal reaction conditions (*i.e.* total volumes of reactions, primers, concentrations of DNA polymerase, incubation temperatures and times, template DNA, as well as $MgCl_2$) were optimized for each DNA fragment.

1. To a sterile thin wall PCR tube on ice, add and mix the following components:

   5 µl 10× PCR buffer (includes $MgCl_2$)

5 μl dNTP mixture (Invitrogen, 2 mM)

1.5 μl Sense primer (20 μM)

1.5 μl Anti-Sense primer (20 μM)

1 ng Template plasmid (or a bacterial colony)

0.5 μl *Taq* DNA polymerase (MBI Fermentas)

Autoclaved distilled water, up to 50 μl per reaction

2. Cap the tube and centrifuge briefly. Put the sample in the PCR thermocycler.
3. Perform 25-30 cycles of PCR amplification as follows:

   Denature at 94ºC for 30 sec

   Anneal at 52-55ºC for 30 sec

   Extend at 72ºC for 1 min (generally 1 min per kb being amplified).
4. Incubate the sample at 72ºC for an additional 5 min and maintain the reaction at 4ºC. The sample can then be stored at -20ºC.
5. Visualize the amplification products by agarose gel electrophoresis with ethidium bromide and DNA molecular weight standards (MBI Fermentas).
6. Purification of the amplified DNA products from the agarose gel was performed by QIAEX II Gel Extraction Kit (Qiagen), according to the manufacturer's protocol.

2.2.5 Mutagenesis of specific Serine residues

The QuickChange Site-Direct Mutagenesis kit was used to generate the Bcl-xL(S62A), Bcl-xL(S106A) and Bcl-xL(S228A) serine-to-alanine substitutions (blocks the ability to be phosphorylated on these serines).

Using Bcl-xL wildtype cDNA as a template, all three pairs of primers (forward and

reverse: substituted codons are underlined, see below) for single amino acid mutations were designed according to the manufacturer's specifications. All primers are designed as follow:

Bcl-xL(S62A)

Forward: 5'-GCA CCT GGC AGA CGC CCC CGC GGT GAA TGG-3'

Reverse: 3'-CGT GGA CCG TCT GCG GGG GCG CCA CTT ACC-5'

Bcl-xL(S106A)

Forward: 5'-GTA CCG GCG GGC ATT CGC TGA CCT GAC ATC CCA GC-3'

Reverse: 3'-CAT GGC CGC CCG TAA GCG ACT GGA CTG TAG GGT CG-5'

Bcl-xL(S228A)

Forward: 5'-TTC TGC TGG GCG CAC TCT TCA GTC GG-3'

Reverse: 3'-AAG ACG ACC CGC GTG AGA AGT CAG CC-5

The PCR-based procedure for mutagenesis is as follow:

1. Add all reagents (except primers, all are from Stratagene) in an autoclaved PCR tube:

10×Reaction buffer	5 μl
Plasmid cDNA template (50 ng)	2 μl
Forward primer (125 ng/μl)	1 μl
Reverse primer (125 ng/μl)	1 μl
dNTP mix	1 μl
ddH_2O	40 μl

2. Vortex tube and add 1 µl of *Pfu* Turbo DNA polymerase 2.5 U/µl (Stratagene).

3. Place the tube into the PCR machine (thermocycler) and amplify the cDNA using the following program:

First step:	1 cycle:	95ºC for 30sec
Second step:	16 cycles:	95ºC for 30sec
		55ºC for 1min
		68ºC for 6min 30sec
Third step:		4ºC, overnight.

4. Put the PCR tube back on ice for 2 min.

5. Add 1 µl of 10 U/µl *Dpn I* (Stratagene) into PCR tube and mix well to digest the methylated template plasmid cDNA.

6. Incubate at 37ºC for 1 hour.

7. Defrost XL1-Blue super competent cell (Stratagene) on ice.

8. Combine 1 µl of PCR-amplified plasmid cDNA with 50 µL of competent cell in a 15 ml Falcon tube on ice for 30 min.

9. Heat shock the mixture at 42ºC for 45 sec.

10. Put the tube back on ice for 2 min and add 500 µl of NYZ^+ (10 g NZ amine casein hydrolysate, Sigma; 5 g Bacto-Yeast extract, BD Bioscience; 85.5 mM NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, EMD Chemicals Inc; 20 mM glucose, BDH; pH 7.5) preheated to 42ºC.

11. Incubate the tube in the incubator/shaker at 37ºC for 1 hour at 250 RPM.

12. Spread bacterial culture on DYT/Amp^+ plate; incubate at 37ºC for 16-18 hours.

13. On the following day, pick colonies (ampicillin resistant) and screen for the

plasmid/cDNA insert using colony PCR.

2.2.6 Mini-prep

Mini-prep'ing is a process for amplifying small amounts of plasmid DNA for sequencing or colony screens. The mini-prep procedure is as follows:

a) Bacterial growth and cell harvesting

1. Transfer a bacterial colony into 5 ml of Terrific Broth medium (TB, 12 g Bacto-Tryptone, BD Bioscience; 24 g Bacto-Yeast extract, BD Bioscience; 4 ml glycerol, MP Biomedicals; 900 ml ddH_2O; autoclaved) containing 0.5 ml of 0.17 M KH_2PO_4/0.72 M K_2HPO_4 (J.T. Baker) and add ampicillin to the solution (final concentration: 50 μg/mL).
2. Incubate overnight at 37ºC, 250 RPM in bacterial shaker.
3. Take 1.5 ml of culture and centrifuge at 12000*g* for 30 sec at room temperature.
4. Store the remainder culture at 4ºC and remove supernatant by aspiration.

b) Lysis by alkali

1. Add 100 μl of ice-cold Solution I (50 mM Glucose; 10mM EDTA, pH 8.0; 25 mM Tris (base), pH 8.0) and resuspend bacteria by vigorous vortexing.
2. Add 200 μl of Solution II (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS)) and mix content gently by inverting tube back and forth 10 times and set tube on ice for 5 min.
3. Add 150 μl of ice-cold Solution III (5 M potassium acetate solution, pH 4.8) mix gently by rapid inverting tube. Set the tube on ice for 5 min.
4. Centrifuge tube at 12000*g* for 5 min at 4ºC, transfer supernatant to a new tube.

5. Add 450 µl of phenol (Sigma): Chloroform (BDH) and mix well.
6. Centrifuge tube at 12000*g* for 2 min and transfer top phase to a new tube.
7. Precipitate double-strand DNA with 2 volumes of 100% ethanol (EMD Chemicals Inc) at room temperature for 2 min.
8. Centrifuge at 12000*g* for 10 min at room temperature and remove supernatant.
9. Wash DNA with ice-cold 70% ethanol and centrifuge at 4ºC (12000*g* for 10min).
10. Remove excessive liquid and speed-vacuum for 20 min.
11. Re-dissolve the pellet in 40 µl of TE pH8.0 (10 mM Tris, 1mM EDTA) containing DNAse-free pancreatic RNAse (20 µl/ml).
12. Incubate at 20ºC for 20 min, can be stored at -20ºC.
13. Dilute plasmid DNA at 1:80 (stock:H_2O), measure DNA concentration by UV-spectrometry at an absorbance of 260 nm.
14. (For sequencing, take 5 µl of 4 µM CMV primer (from 100 µM stock) and 5µl of 100 ng/µl plasmid DNA.)

2.2.7 Maxi-prep

Maxi-prep'ing is a large-scale process for amplifying and purifying plasmid cDNA. The procedure is similar to mini-prep.

a) Bacterial growth and cell harvesting

1. Transfer a bacterial colony into 5 ml of Terrific Broth medium containing 0.5 ml of 0.17 M KH_2PO_4/0.72 M K_2HPO_4 (J.T. Baker) and add ampicillin (final: 50 µg/mL).
2. Incubate overnight at 37ºC, 250 RPM in bacterial shaker.
3. Combine 5 ml of bacterial culture with 270 ml TB and 30 ml 0.17 M KH_2PO_4/ 0.72 M

K_2HPO_4 in a 1L conical flask (make sure the culture contains ampicillin).

4. Incubate the conical flask at 37ºC, 250 RPM in bacterial shaker, growth 16-18 hours.
5. The next day centrifuge cells at 4ºC, 5000 RPM 15 min, remove supernatant.

b) Lysis of cells

1. Add 10 ml of solution I resuspend cells
2. Add 20 ml of solution II mix gently by inverting bottle back and forth 5 times.
3. Store at room temperature for 5-10 min.
4. Add 20 ml solution III and mix well, store on ice for 5 min.
5. Centrifuge for 15 min at 5000*g* at room temperature.
6. Filter supernatant through 4 layer of cheesecloth into a new 250 ml centrifuge bottle.
7. Add 48 ml of isopropanol (EMD Chemicals Inc) and mix well. Then keep at room temperature for 1 hour.
8. Centrifuge for 15 min at 5000*g* at room temperature.
9. Remove supernatant and rise with 85% ethanol, air dry and then dissolve pellet in 3 ml TE.

c) Plasmid DNA purification

1. Pour DNA solution (from #9, above) into a 15 ml tube, add 4.8 ml lithium chloride (LiCl, J.T. Baker).
2. Centrifuge at 9000*g* for 10 min at room temperature.
3. Transfer supernatant to a new tube. Add 7.8 ml of isopropanol and centrifuge at 9000*g* for 10 min at room temperature.

4. Remove supernatant and rinse pellet with 85% ethanol, air-dry and dissolve pellet in 500 µl TE with 5 µl of RNAse (10 mg/ml).
5. Incubate at room temperature for 30 min.
6. Add 400 µl of 1.6 M NaCl with 13% (w/v) PEG8000, mix and centrifuge at 12000*g* for 2 min at 4ºC.
7. Remove supernatant and dissolve pellet in 500 µl TE.
8. Add 500 µl phenol, vortex and centrifuge at 4000*g* for 5 min at room temperature.
9. Transfer top phase to a new 1.7 mL Eppendorf tube and extract with phenol again.
10. Add 500 µl chloroform, vortex, and centrifuge at 4000*g* for 5 min at room temperature.
11. Transfer top phase to a new Eppendorf tube, add 5 M NaCl to a final concentration of 125 mM.
12. Add two volumes of 400 µl ice-cold ethanol incubate on ice for 5 min.
13. Centrifuge for 5 min at 12000*g*, 4ºC.
14. Remove supernatant and add 200 µl of 85% ethanol, vortex and centrifuge at 12000*g* for 2 min at 4ºC.
15. Remove supernatant and air dry ethanol.
16. Dissolve pellet in 300-500 µl nano-H_2O.
17. Adjust plasmid DNA concentration to 1 µg/µl, store at -20ºC.

2.2.8 Transfection of mammalian cultures

Transformation of cultured eukaryotic cells with foreign DNA is often called transfection. Lipofectamine 2000 (introduces plasmid DNA into cell cultures by lipofection) was the transfection reagent used during the current thesis work.

In this study, HEK293A cells and PC12 cells were transiently transfected (allowed to express protein for 24 hours or 48 hours). The procedures are as follows:

1. Use serum-free culture medium to dilute DNA.
2. Use serum-free culture medium to dilute Lipofectamine2000 regent.
3. Combined two reagents and incubate at room temperature for 20 min.
4. Add DNA-Lipofectamine2000 mixture to cell cultures.
5. Change to fresh culture medium 4 hours post-transfection (this minimizes toxicity associated with the transfection reagent).
6. Incubate cells for 24 or 48 hours at 37ºC with 5% CO_2 in cell culture incubator.
7. After transfection, cells were harvested and washed with 1×PBS, centrifuge at 1000g for 5 min at 4ºC. remove supernatant and Lysis with Lysis buffer (1% Triton-X100, Sigma; 20mM Tris pH 7.5, ICN Biochemicals; 10% glycerol, MP Biomedicals; 1mM EDTA, EMD Chemicals Inc.) containing 100× protease inhibitor cocktail (PIC, Sigma) incubated on ice for 30 min, then centrifuge at 16,100*g* at 4ºC for 20 min.
8. Determine each cell lysate protein concentration by BCA™ protein assay kit(Pierce).

2.2.9 Western blot analysis

Western blotting, also known as immunoblotting (because antibodies are used as the primary detection tool), is a technique used to detect a specific protein expression in a cell extract (or tissue homogenate). Equal amounts of denatured total protein from cell lysates are loaded onto an SDS-polyacrylamide gel. Using electrophoresis, proteins migrate along the current and are separated (resolved) according to their molecular weight, *i.e.* the smaller the protein, the faster it will migrate. Proteins are then transferred from the gel to a nitrocellulose

or PVDF membrane and the specific protein (now on the membrane) is detected by specific primary antibody. This primary antibody is then detected by secondary antibody conjugated with horseradish peroxidase. The horseradish peroxidase converts a chemiluminescence substrate that can be detected on photographic film.

1. Prepare SDS-PAGE gel (1mm thick), which includes a 12% resolving gel and 4% stacking gel, as shown in Table 2.3.
2. Protein samples are denatured (to their linear structure) by boiling at 100ºC with 4× Laemmli sample buffer (LB, 8% SDS, 40% glycerol, 10% mercaptoethanol, 0.02% bromophenol blue, 0.25 M Tris-HCl pH 6.8).
3. Load protein ladder into one lane and in other lanes load equal amounts of protein, up to 40 µl per well. Electrophorese at 110V in 1×running buffer (25 mM Tris-HCl, 0.1% SDS, 250 mM glycine) until bromophenol blue reaches the bottom of the gel.
4. Transfer protein from gel to a 0.22 µm nitrocellulose membrane (Bio-Rad) at 25 V for 90 min in 1×transfer buffer (25 mM Tris-HCl, 0.00375% SDS, 20% methanol, 250 mM glycine, 4ºC).
5. Once transferred, remove the membrane to a container and block the non-specific sites with 5% milk in 1×TBS buffer (with gentle rocking at room temperature for 1 hour).
6. Wash membrane with 1×TBS-Tween buffer (25 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.1% Tween-20, Sigma) for 30 sec and incubate membrane with primary antibody, rocking at 4ºC overnight.
7. Wash the membrane with 1×TBST twice for 10 min per time.
8. Incubate the membrane with HRP-conjugated secondary antibody for 1 hour in 5%

milk with 1×TBST at room temperature.

9. Wash membrane with 1×TBST three times, 10 min per time.

10. Incubate membrane for 1 min with ECL Western Blot Detection Reagent (GE Healthcare) and expose to film to visualize the bands.

2.2.10 Immunoprecipitation of target protein

Immunoprecipitation (IP) is a technique for isolating proteins and for detecting protein-protein interactions. This technique can isolate a specific protein from whole cell lysate for subsequent analysis by standard Western blotting. The pre-clearing step is to lower the non-specific antigen binding with antibody. The procedure is described below:

a) Sample preparation.

1. Transfect cells with plasmid DNA and let overexpress 24 or 48 hours.

2. Treat cells according to the individual experimental design.

3. Harvest and wash cells once with 1×PBS.

4. Lyse cells with Lysis Buffer containing 1×PIC (protease inhibitor cocktail, Sigma) on ice for 30 min.

5. Centrifuge cell lysate at 14000*g* for 20 min and determine protein content.

b) Pre-clearing

1. Take 100-500 μg of protein in 400 μl of Lysis Buffer containing 1×PIC.

2. Add 1-5 μg of Mouse IgG (in a ratio of 1:100, antibody:protein) and 30 μl of sepharose A+G, rocking at 4ºC for 1 hour.

3. Centrifuge at 9000*g* for 10 min, move pre-cleared supernatant to a new tube (350 μl).

Table 2.3

	12% Resolving Gel (10 ml)	**4% Stacking Gel (5ml)**
ddH_2O	3.34ml	3.00ml
Buffer A	2.50ml	-
Buffer C	-	1.25ml
Acrylamide	4.00ml	0.67ml
10% APS	50µl	25µl
10% SDS	100µl	50µl
TEMED	10µl	5µl

Buffer A: 1.5 M Tris-HCl, 0.5% SDS, pH 8.8

Buffer C: 1.5 M Tris-HCl, 0.5% SDS, pH 6.8

Acrylamide (Bio-Rad) and TEMED (Sigma) were stored at 4ºC.

c) Antibody incubation

1. Add 1-5 μg of antibody (1: 100, antibody: protein), rocking at 4ºC overnight.
2. Then add 30 μl of sepharose A+G rocking at 4ºC for 2 hours.
3. Centrifuge at 4ºC at 9000*g* for 10 min to spin down all Sepharose beads.
4. [Supernatant can be collected to run on a gel to show immunodepletion has been achieved.]
5. Wash beads with Lysis Buffer three times and centrifuge at 9000*g* for 5 min each time.
6. After last wash, get rid of excessive liquid left in the tube and add 30 μl of 2× Laemmli sample buffer.
7. Boil at 100ºC for 5 min to denature protein.

d) Load on the SDS-PAGE (gel).

2.2.11 Immunofluorescence and double-staining

Hoechst 33258 (Sigma), DAPI (4, 6-diamidino-2-phenylindole, Invitrogen), MAP2 (Santa Cruz), FLAG (Sigma) and Phospho-Akt Ser473/ Thr308 (Cell Signaling) were used for immunofluorescence staining. Microtubule-associate protein 2 (MAP2) belongs to microtubule-associated protein family and stabilizes microtubule growth by crosslinking other intermediate filament and microtubules. Hoechst 33258 fluorescently labels DNA and was used to examine chromatin condensation. DAPI can bind with double-stranded DNA and forms a fluorescent complex that allows the visualization of the nucleus.

PC12 cells were plated in 8-well chamber slides coated with rat-tail collagen. After the appropriate treatment, cell culture medium was removed and cells were fixed with 4% PFA

for 1 hour and then stored until needed for staining.

The staining procedure is as follows:

1. Wash with 1×PBS 1time for 5 min to get rid of PFA.
2. Permeabilize cells with 0.25% Triton-X100 (2% BSA) in 1×PBS for 1 hour.
3. Add anti-MAP2 primary antibody in 1:250 dilution ratio to each well (125 μl/well) in a dark humid environment; incubate for 1 hour (overnight, if necessary).
4. Rinse with 1×PBS 1time for 5 min.
5. Add secondary antibody Donkey-anti-Rabbit (or anti-Mouse) FITC-conjugate at a 1:200 dilution to each well (200 μl/well) and incubate at room temperature for 2 hours.
6. Rinse with 1×PBS for 5 min.
7. Add Hoechst 33258 (50 μg/ml stock) in a 1:100 dilution ratio or add DAPI (9 mM stock) in a 1:3000 dilution, and incubate in the dark for 5-10 min.
8. Wash with 1×PBS twice for 10 min.
9. Remove all liquid and air dry for 5 min.
10. Add a few drop of Prolong Gold anti-fade medium (Invitrogen) and seal well.
11. Immunodetection was performed on an Olympus FV300 confocal microscope or on an Olympus BX-51 fluorescence microscope.

2.2.12 MTT conversion assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a method to determine mitochondrial function and is often used as a means of determining the toxicity of a potential medical reagent (*i.e.* more toxicity = less mitochondrial function).

Cells were plated in a 96-well plate and grown overnight. After treatment, culture

medium was removed and 50 µl of 0.5 mg/mL MTT (diluted in DMEM medium containing 1% FBS from 5 mg/mL stock) was added. The cells were left to incubate at 37ºC for 2-4 hours. After incubation, 100 µl of DMSO was added to dissolve the purple formazan crystals. MTT conversion/cell viability was tested by quantify the absorbance of the colored solution by Spectra Max plus 384 microplate reader (Molecular Devices) at 570 nm.

2.2.13 Statistics

Significance (set at $P<0.05$) was assessed by unpaired t-test or by one-way ANOVA with *post hoc* analyses relying on Bonferroni's Multiple Comparison Test (GRAPHPAD PRISM v3.01; GraphPad Software Inc., San Diego, CA, USA). Data are represented as mean ± SEM.

3. Results

3.1 Specific Serine residues in pro-apoptotic Bcl-xS dictate its toxicity.

Dr. Zelan Wei, a postdoctoral researcher in our laboratory, originally chose to investigate the role(s) of three Serines in the regulation of pro-apoptotic Bcl-xS function. The three residues were substituted for Alanines so as to "mimic" dephosphorylation and were chosen because: (a) Ser62 is a potential inactivation mutant (homologous to Ser70 in Bcl-2, [30, 36]); (b) Ser106 resides within a putative Akt phosphorylation motif (R*XX*R*XX*S); and (c) Ser165 lies immediately upstream of the hydrophobic tail (homologous to Ser184 in Bax, which is known to influence the mitochondrial localization and toxicity of Bax, [89]). This same justification applies to the substitution of Ser62, S106 and S228 in Bcl-xL (examined later).

In collaboration with Dr. Wei, the three Bcl-xS Ser-to-Ala variants were expressed in PC12 cells and cytotoxicity was determined using MTT conversion. Of the three mutations, only Bcl-xS(S106A) and Bcl-xS(S165A) decreased PC12 cell viability (Fig. 3.1). The expression of Bcl-xS variants was difficult to accomplish in PC12 cells (Fig. 3.1, bottom panels), yet they were still able to exert cytotoxic effects.

The three Bcl-xS Ser-to-Ala variants were also expressed in HEK cells (often used for signalling studies because of their capacity for overexpressing proteins). The three variants expressed well in HEK cells (Fig. 3.2, bottom panels) and all three decreased HEK cell viability (again determined using MTT conversion) (Fig. 3.2).

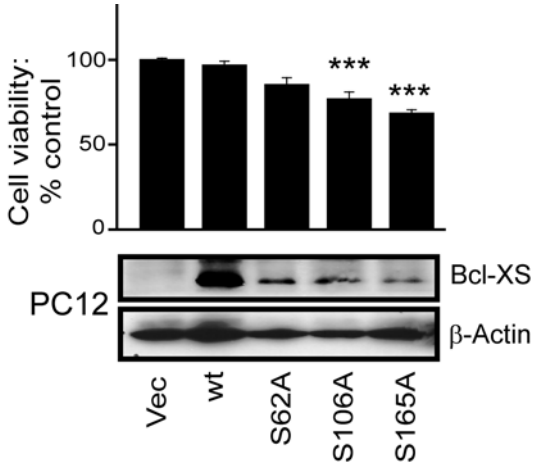


Figure 3.1: Overexpression of Bcl-xS Ser-to-Ala variants affects PC12 cell viability. Bcl-xS S62A, S106A and S165A variants were overexpressed in PC12 cell cultures (*lower panels*, β-actin was included to demonstrate equal protein loading). Cell viability was determined 24 hours post-transfection using the MTT conversion assay. Only the S106A and the S165A variants decreased PC12 cell viability [$F_{(4,29)}$=25.06, ***P<0.001]. (Used with permission, ref [44]).

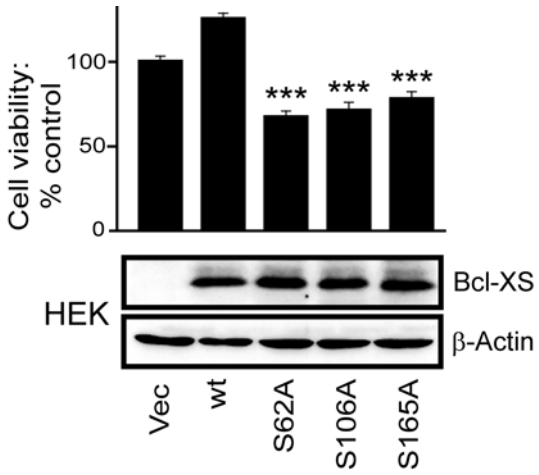


Figure 3.2: Overexpression of Bcl-xS Ser-to-Ala variants affects HEK cell viability. Bcl-xS S62A, S106A and S165A variants were overexpressed in HEK cell cultures and cell viability (MTT conversion) was determined 24 hours post-transfection. All three variants expressed very well (*lower panels*, β-actin was included to demonstrate equal protein loading) and all three variants decreased HEK cell viability [$F_{(4,29)}$=32.72, ***P<0.001]. (Used with permission, ref [44]).

For the remainder of these studies, we chose to focus on the Bcl-xS(S106A) variant as the working hypothesis is that Ser106 is *a novel target for Akt* as it resides in a putative Akt consensus motif.

Bcl-xS(S106A) was overexpressed in PC12 cells as well as in HEK cells for 24 hours. Cells were then harvested and homogenized. Subcellular fractions (mitochondrial and cytosolic) were then collected by differential centrifugation. In PC12 cells, the overexpression of Bcl-xS(S106A) resulted in the release of cytochrome *c* (cyto *c*) from the mitochondrial to the cytosolic fraction (Fig. 3.3, left side). The expression of VDAC was similar in both vector-transfected (control) and Bcl-xS(S106A) mitochondrial fractions. Examination of TCL revealed that PARP was cleaved to the p85 fragment (this cleavage is known to be a caspase-3-mediated event, [90]). In HEK cells, the overexpression of Bcl-xS(S106A) resulted in a different pattern of events (Fig. 3.3, right side). First, cyto *c* was not released from the mitochondrial fraction. Secondly, the expression of VDAC was significantly diminished in the Bcl-xS(S106A) extracts and, finally, PARP was not cleaved to the p85 fragment.

So as to determine if the effects of Akt on Bcl-xS function could be being mediated directly through the Ser106 residue, co-immunoprecipitation experiments were performed. In this case, the Bcl-xS proteins were overexpressed in HEK cells (because they express proteins so effectively, Fig. 3.4a) and the FLAG-tag was used to specifically immunoprecipitate the Bcl-xS WT or S106A proteins from precleared lysates. The isolated immunoprecipitates were then resolved by SDS-PAGE and blots were probed for specific proteins. FLAG-Bcl-xS(S106A) co-immunoprecipitated with VDAC better did the FLAG-Bcl-xS WT protein (Fig. 3.4a) suggesting that the S106A protein was localizing preferentially to the mitochondrial fraction. In a separate set of experiments it was demonstrated that the FLAG-Bcl-xS WT co-

immunoprecipitated better with the pSer473-Akt protein (an activated form of Akt) than did the FLAG-Bcl-xS(S106A) variant (Fig. 3.4b). In support of this observation, similar protein extracts were immunoprecipitated with the pSer473-Akt antibody and the SDS-PAGE resolved proteins were examined for FLAG. Again, a better co-immunoprecipitation was observed in extracts expressing FLAG-Bcl-xS (Fig. 3.4b). These data suggest that disruption of Ser106 allows Bcl-xS to localize to the mitochondria, where it interacts with VDAC. This coincides with its inability to co-immunoprecipitate with activated Akt, thus supporting the hypothesis that Ser106 in Bcl-xS is a target for cytoplasmic Akt.

At this point, I chose to undertake the following experiments with the goal of determining whether the homologous Serines in the anti-apoptotic protein Bcl-xL, *e.g.* Ser62, Ser106 & Ser228, contributed to overall protein function and to cell viability/phenotype.

3.2 Subcloning of Bcl-xL WT and substitution mutants.

The human Bcl-xL cDNA obtained from the Youle Laboratory was confirmed using a BLAST search of the NCBI GenBank database. Bcl-xL wildtype (WT) cDNA was then subcloned into pCMV/FLAG3 (Fig. 3.5). The restriction sites used for subcloning were *Bgl* II and *EcoR* I (see section 2.2.2 for protocol, Fig 3.5 and Fig 3.6). The pCMV/FLAG3-Bcl-xL construct (Fig 3.5) was sequenced with the CMV primer (Fig 3.7). pCMV/FLAG3-Bcl-xL WT was then used to generate the S62A, S106A and S228A substitutions (Fig. 3.8), based on homologous Serine-to-Alanine substitutions we had used to study the effect of Serine [de]phosphorylation on the pro-apoptotic homologue Bcl-xS [44].

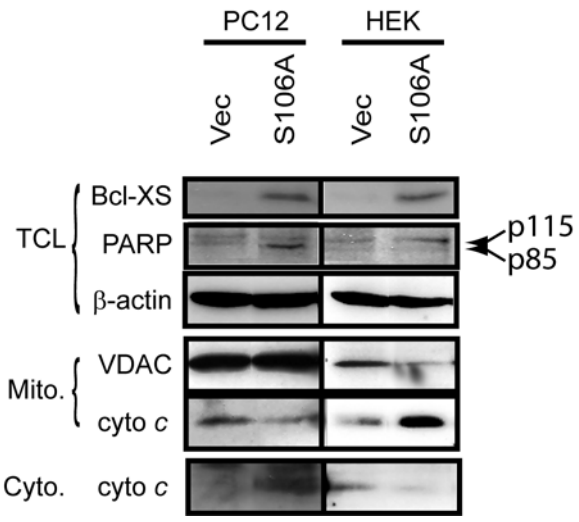


Figure 3.3: Bcl-xS(S106A) exerts different effects on mitochondrial function in PC12 and HEK cells. The Bcl-xS(S106A) variant was overexpressed in PC12 cells as well as in HEK cells for 24 hours. Cells were then harvested for subcellular fractionation studies. Bcl-xS overexpression is shown in total cell lysates (TCL) (β-actin was included to demonstrate equal protein loading). PARP expression and cleavage (as an indicator of apoptotic cell death) was also examined. Note that PARP is cleaved to the p85 fragment in PC12 cells, but not in the HEK cells. Furthermore, in PC12 cells, cytochrome *c* (cyto *c*) is released from the mitochondria (Mito.) into the cytosol (Cyto.). Levels of VDAC, a mitochondrial protein important for transport of proteins, including cyto *c*, across the mitochondrial membranes is unaffected by ectopic Bcl-xS expression in PC12 cells. In contrast, cyto *c* remains mitochondrial in HEK cells and the expression of VDAC is significantly reduced. (Used with permission, ref [44]).

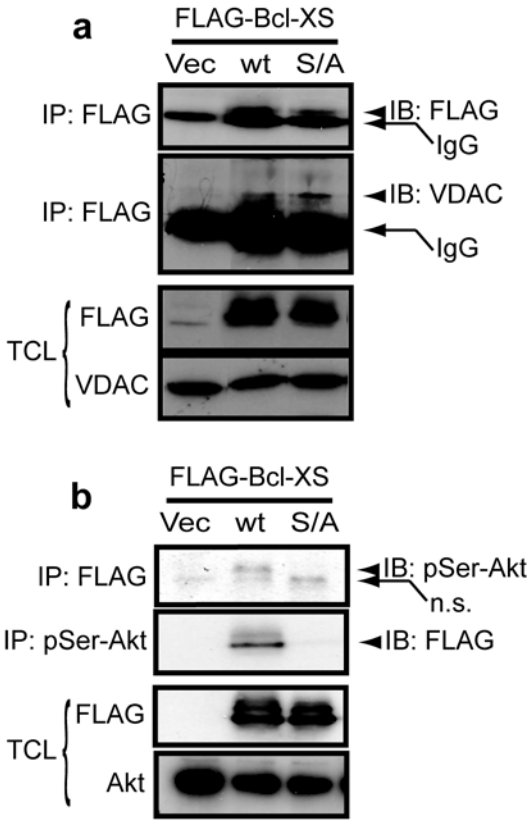


Figure 3.4: The Bcl-xS(S106A) substitution influences the interaction with VDAC and with Akt. FLAG-tagged Bcl-xS WT and Bcl-xS(S106A) (S/A) were overexpressed in HEK cells. 24 hours later the cells were harvested and pre-cleared cell lysates were then immunoprecipitated with the FLAG antibody. (**a**) The corresponding SDS-PAGE resolved proteins were probed for FLAG and VDAC. FLAG-Bcl-xS(S106A) interacted more with VDAC than did the FLAG-Bcl-xS WT. The overexpression of the FLAG-tagged proteins and the expression of endogenous VDAC were confirmed in total cell lysates (TCL). (**b**) In a separate experiment, pSer473-Akt was shown to immunoprecipitate with FLAG-Bcl-xS, but not the S/A variant, while the complementary experiment shows that FLAG-Bcl-xS WT immunoprecipitates better than FLAG-Bcl-xS(S106A) with pSer473-Akt. FLAG overexpression and Akt expression were examined in TCL. IgG: light chain of the immunoprecipitating antibody, n.s.: non-specific band detected at 50kDa.

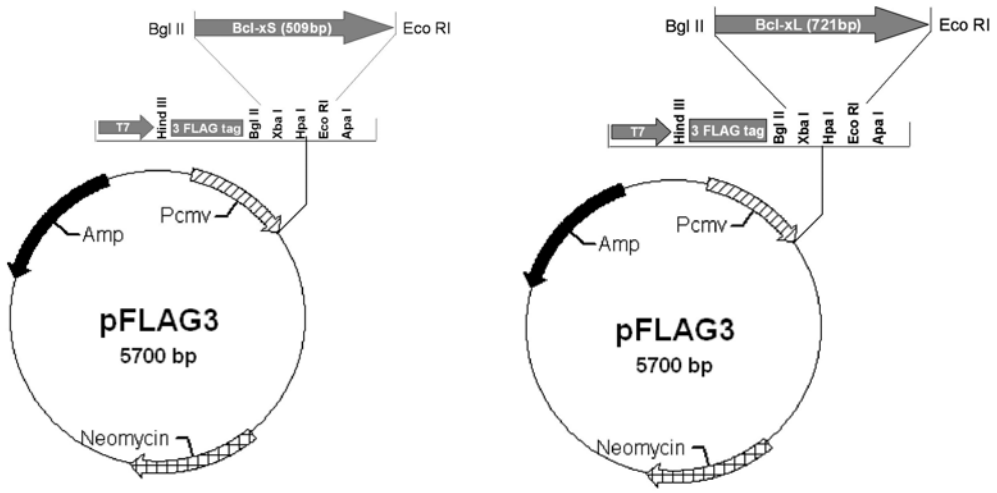


Figure 3.5: Plasmid map of pCMV/FLAG3-Bcl-xS and pCMV/FLAG3-Bcl-xL. The pCMV/FLAG3 plasmid is about 5700 basepair (bp). The Bcl-xS (509 bp) and Bcl-xL (721 bp) cDNA are inserted using the *Bgl*II and *Eco*RI restriction sites. "Amp" indicates ampicillin resistance and is used as a selection marker for positively transformed bacteria. Neomycin is used for eukaryote screening (not used in the current thesis work).

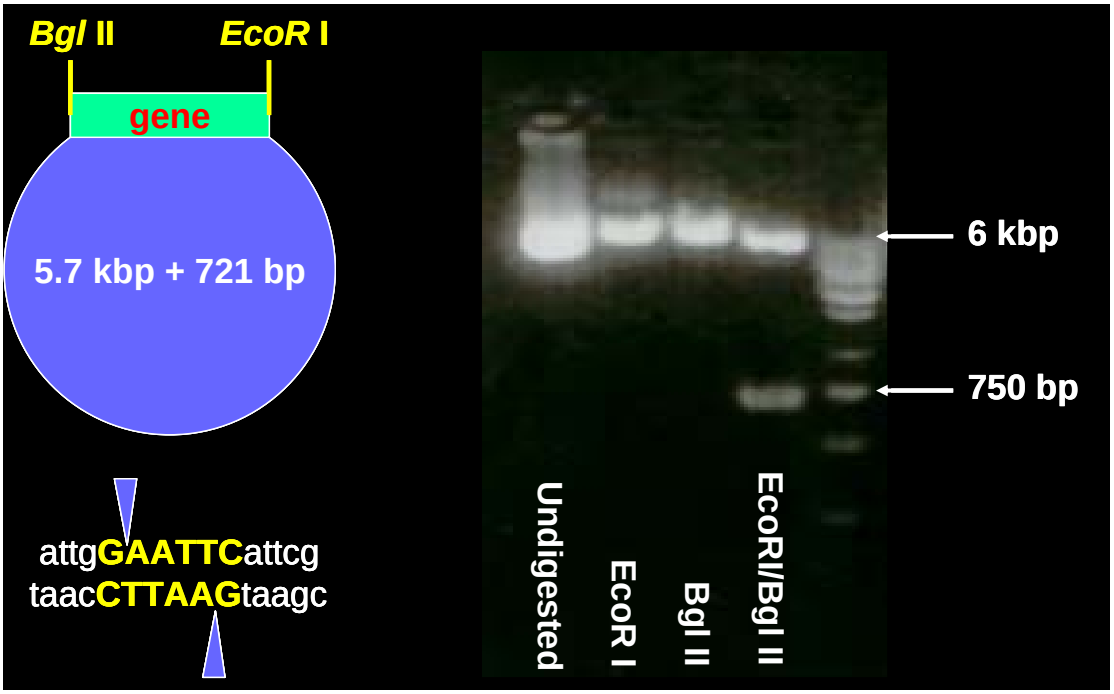
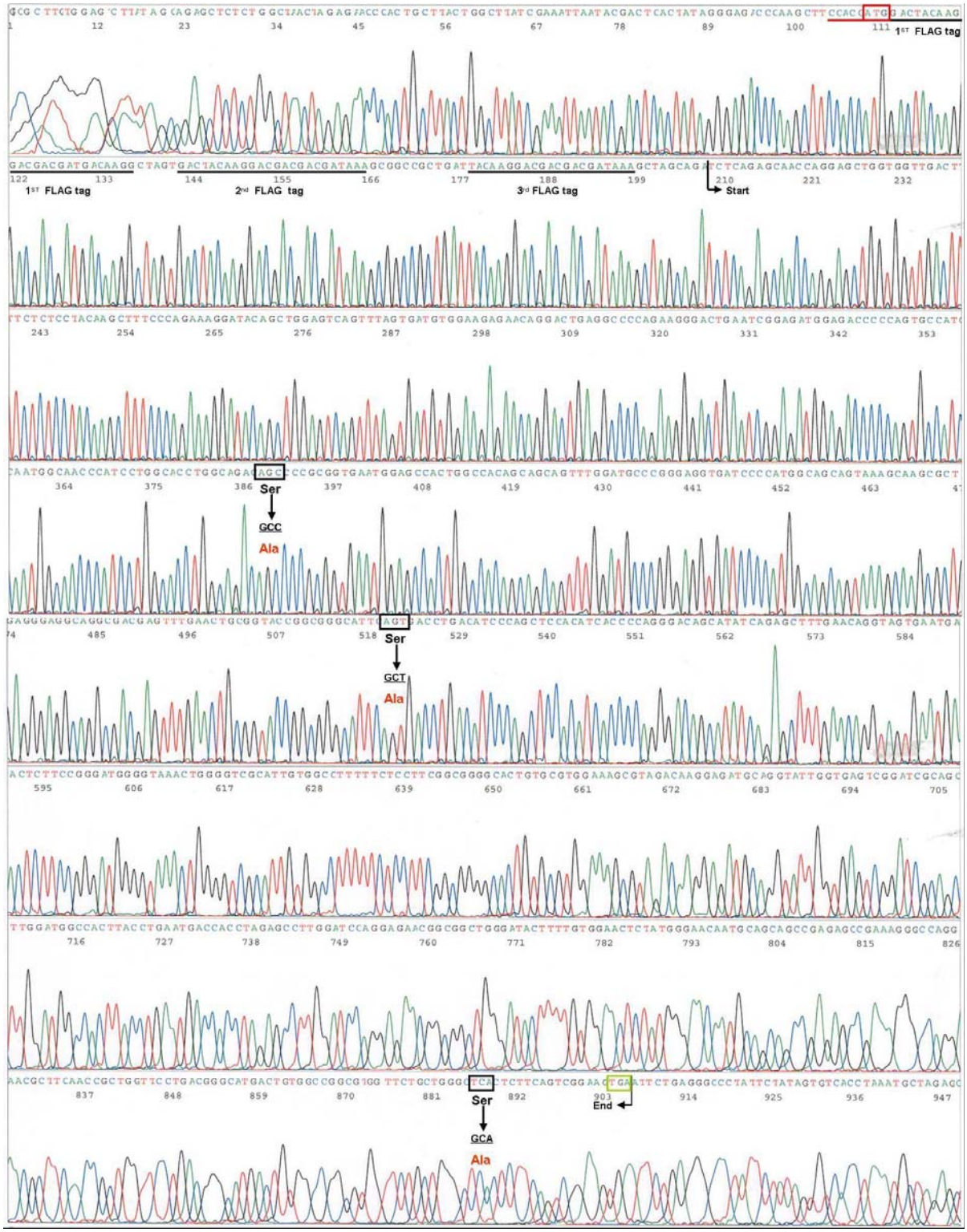


Figure 3.6: Confirmation of the Bcl-xL cDNA insert in pCMV/FLAG3 using restriction analysis. (left) The Bcl-xL WT cDNA was subcloned into the *Bgl*II and *EcoR*I restriction sites in pCMV/FLAG. The nucleotide sequence GAATTC (indicated in yellow) is the palindromic sequence recognized by *EcoR*I. (right) The pCMV/FLAG3-Bcl-xL plasmid was restricted with either *Bgl*II or *EcoR*I, or with both enzymes, and the reaction was then electrophoresed on agarose gel and visualized using ethidium bromide. The single restrictions simply linearized the plasmid (*i.e.* it migrates as a more distinct band at approximately 6.4 kbp. In contrast, the double-digested plasmid (*EcoR*I/*Bgl*II) releases a 721 bp fragment and an approximate 6 kbp fragment (empty vector).


1ST FLAG tag
1ST FLAG tag
2nd FLAG tag
3rd FLAG tag
Start
Ser
GCC
Ala
Ser
GCT
Ala
End
Ser
GCA
Ala

(Previous page)

Figure 3.7: The chromatogram covering the Bcl-xL WT cDNA sequence. pCMV/FLAG3-Bcl-xL was sequenced using the CMV primer (this anneals with the "CMV promoter" of the pCMV/FLAG3 vector (see Fig. 3.5). The three FLAG epitope coding sequences are indicated (underlined in black) and are found upstream, and in-frame, with the human Bcl-xL cDNA. The start of Bcl-xL cDNA is indicated by the "start" arrow. Note that there is no start "ATG" codon for the Bcl-xL cDNA as it does not require one; it uses the start "ATG" (red box) and the Kozak consensus sequence (red, underlined) immediately upstream of the first FLAG epitope sequence. The end of the Bcl-xL cDNA is indicated by the "TGA" stop codon (green box) and the "end" arrow. The wildtype codons corresponding to Ser62, Ser106 and Ser228 are indicated (black boxes) and the codons used for substituting the Serines for Alanines (Ala) are shown immediately below each Serine.

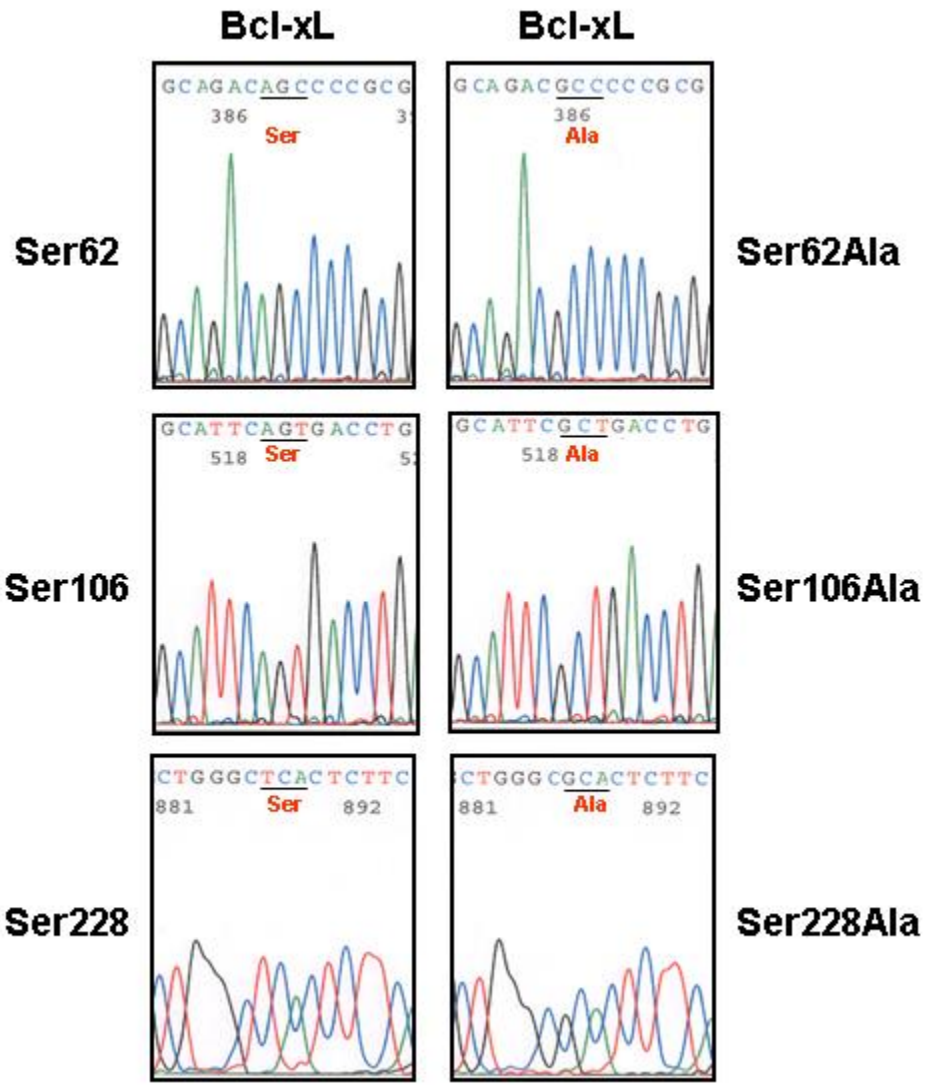


Figure 3.8: Sequencing confirms the Bcl-xL S62A, S106A and S228A mutations. The codon substitution corresponding to the individual Bcl-xL serine-to-alanine (Ser/Ala) mutations in pCMV/FLAG3-Bcl-xL are underlined and labeled "Ala" (shown on the right) and compared to the corresponding wildtype codons (underlined and labeled "Ser", shown on the left side).

3.3 Specific Serine residues in anti-apoptotic Bcl-xL dictate its function.

FLAG-Bcl-xL WT or the three Ser-to-Ala variants were individually expressed in PC12 cells for 48 hours and confirmed by Western blot analysis (Fig. 3.9). The cleavage of the initiator caspase-9 to the active 17 kDa fragment was observed in corresponding cell lysates as was the cleavage of PARP to the p85 fragment (Fig. 3.9). Note that cleavage of the initiator caspase-9 was used to demonstrate a caspase-dependent event in these [PC12] cells (in contrast to the executioner caspase-3 used for studies based on Staurosporine-mediated effects in primary cell cultures, *see* Fig. 3.10, below) as caspase-3 is very difficult to detect (with any reliability) in PC12 cells.

3.4 Staurosporine-induced toxicity is Akt- and caspase-dependent in primary neuronal cultures.

Staurosporine, a potent kinase inhibitor, induced primary neuronal cell death in a concentration-dependent manner (Fig. 3.10a). Staurosporine did not affect the expression of Bcl-xL, but it did inhibit Akt phosphorylation [as evidenced by decreased phosphorylation on both Thr308 and Ser473, while Total-Akt remained the same] (Fig. 3.10a). This coincided with the appearance of indicators of apoptosis, including the 17 kDa active fragment of the executor caspase-3 and cleavage of the caspase-3 targets, i.e. PARP (Poly-ADP ribosome polymerase) and Fodrin (a membrane-associated cytoskeletal protein) (Fig. 3.10b). This suggests that staurosporine-induced primary neuronal death is an Akt- and caspase-dependent event.

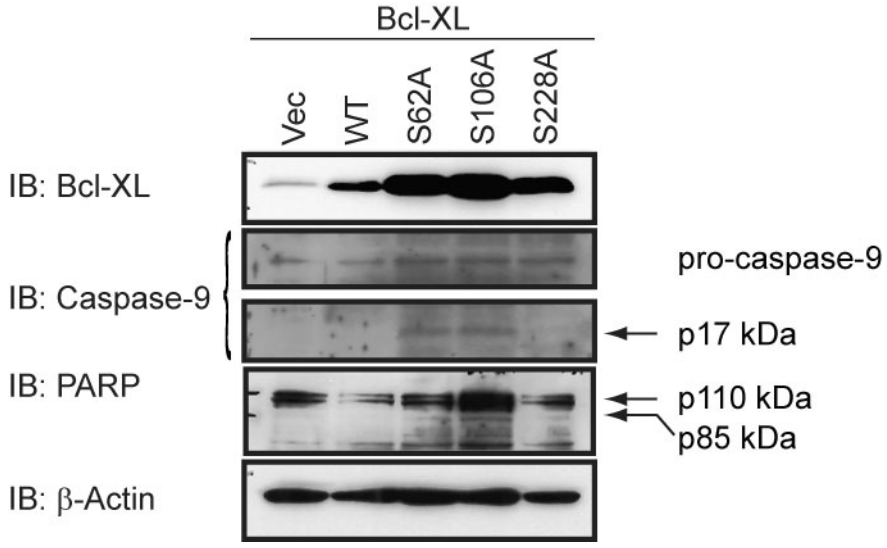


Figure 3.9: Dephosphorylation of selected serine residues of anti-apoptotic Bcl-xL reveals a cytotoxic profile. PC12 cells were transfected with FLAG-Bcl-xL (wildtype and the three Serine-to-Alanine substitution variants, S62A, S106A & S228A) for 48 hours. The overexpression of FLAG-Bcl-xL(S62A) and FLAG-Bcl-xL(S106A) promote the cleavage of the effector caspase-9 (to the p17 (17 kDa) fragment) and the concurrent cleavage of the downstream target, PARP (to the p110 and p85 fragments).

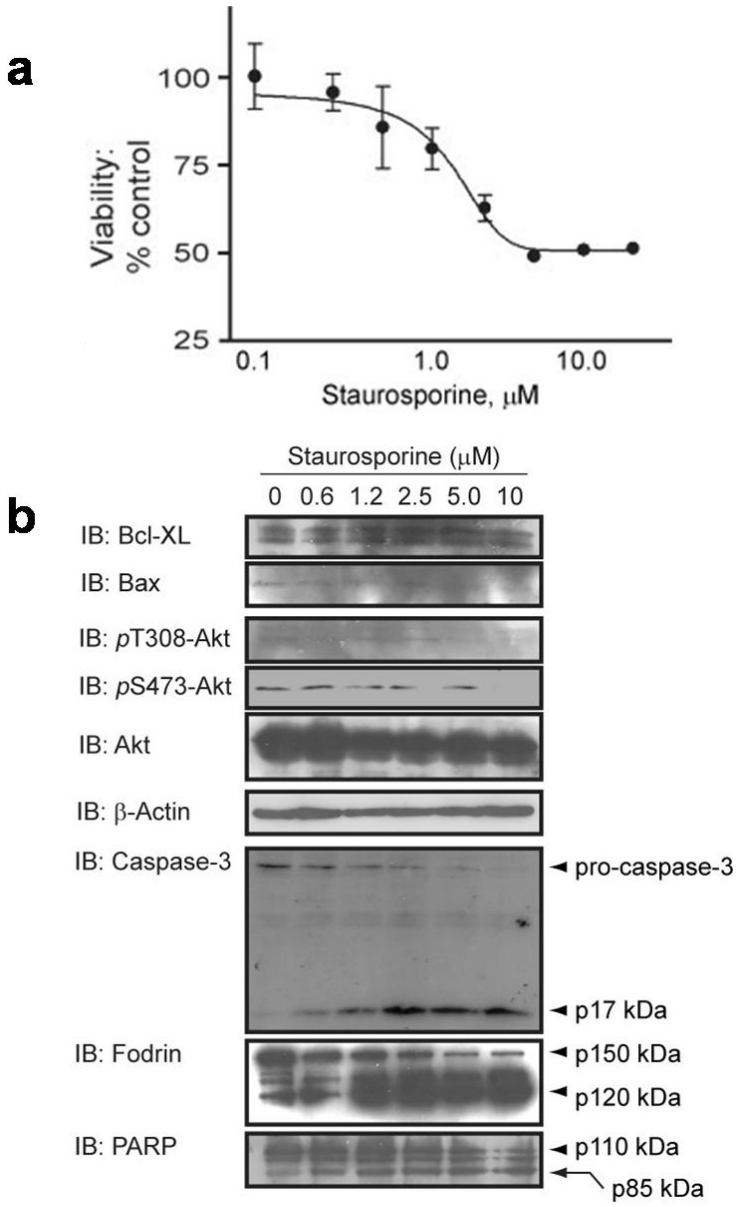


Figure 3.10: Staurosporine-induced primary cell toxicity is Akt- and caspase-dependent. (**a**) Primary cortical cultures were treated with different concentrations of staurosporine for 24 h. Cytotoxicity was demonstrated using the MTT conversion assay. (**b**) Staurosporine did not affect Bcl-xL expression, but it did induce the loss of Akt phosphorylation on both Thr308 and Ser473. It also induced the cleavage of caspase-3 to the active 17 kDa fragment and this coincided with the cleavage of the caspase-3 targets Fodrin (to the 120 kDa fragment) and PARP (to the 85 kDa fragment).

3.5 Staurosporine-induced toxicity in PC12 cells presents with indicators of apoptosis.

Staurosporine (ST) exerted the same effects in PC12 cells as seen with primary neuronal cultures. For example, it inhibited Akt phosphorylation on both Thr308 and Ser473 in a concentration manner (Fig 3.11a) and induced Hoechst-visualized nuclear condensation (a marker of apoptosis) (Fig 3.11b).

Based on the findings using manipulations of the Bcl-xS protein and as the working hypothesis is that Ser106 resides in a putative Akt consensus motif, I chose to focus the following experiments on the Bcl-xL(S106A) variant.

MTT conversion was used to determine how Bcl-xL wt and S106A (24 h expression) affected PC12 cell function during treatment with the potent apoptosis inducer ST (1 μM; 24 h). ST had a significant effect on MTT conversion, but this was not affected by overexpression of either of the proteins (Fig. 3.12). Furthermore, Bcl-xL(S106A) tended to decrease MTT conversion, although this was not statistically significant. The decrease in MTT conversion in these cultures, however, meant that the effect of ST in corresponding cultures was no longer significant.

To determine potential interactors with Bcl-xL during ST treatment, PC12 cells overexpressing FLAG-tagged Bcl-xL wildtype and S106A (for 24 h) were treated with 1 μM ST for an additional 24 hour. Protein extracts were immunoprecipitated with the anti-FLAG antibody and then SDS-PAGE resolved-proteins were examined for FLAG and for Bax (a

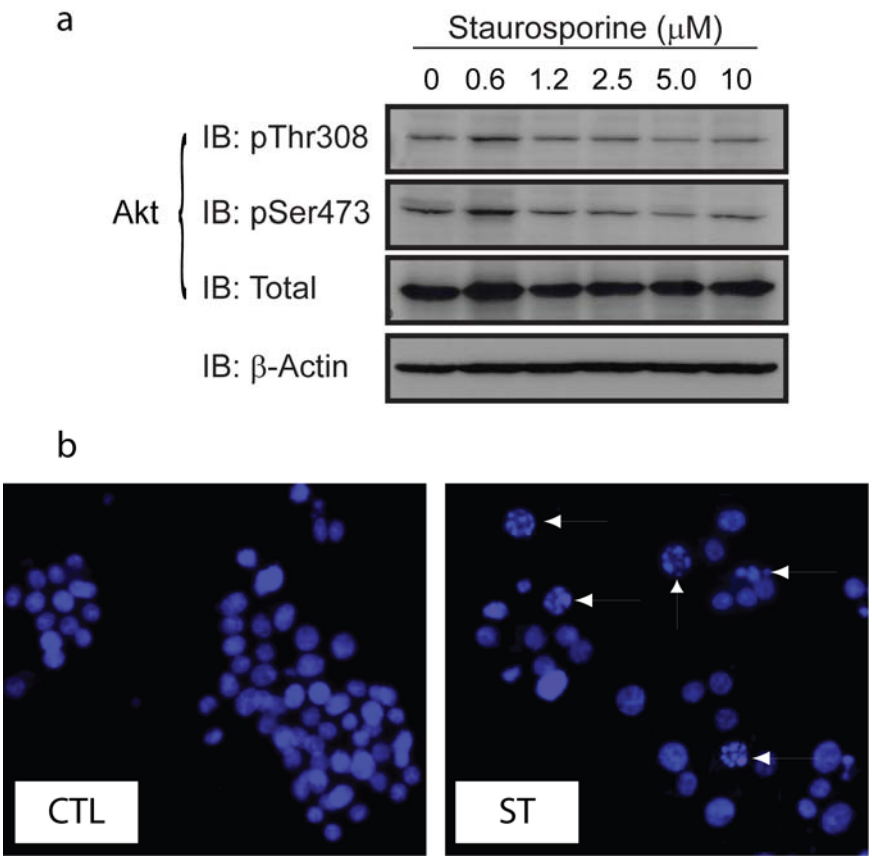


Figure 3.11: Staurosporine-induced PC12 cell apoptosis. (**a**) Akt phosphorylation on Thr308 and Ser473 in PC12 cell lysates is diminished modestly with increasing concentrations of staurosporin. β-Actin was included to demonstrate equal protein loading. (**b**) PC12 cell nuclear condensation (the nucleus appears broken into small clusters; arrows) in cultures treated with 5 μM staurosporine was observed using Hoechst staining.

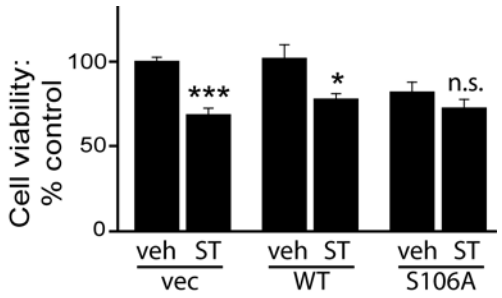


Figure 3.12: Overexpression of Bcl-xL WT and S106A proteins does not alter the staurosporine-induced loss of MTT conversion in PC12 cells. PC12 cells were transfected with Bcl-xL wildtype or S106A. 24 hours later the cells were treated with 5 μM of staurosporine (ST) for an additional 24 hours. Bcl-xL wildtype did not significantly affect the ST-induced decrease in MTT conversion. While the S106A variant tended to decrease MTT conversion, this was not significant. However, the effect of ST in Bcl-xL(S106A)-expressing cultures was also no longer significant. *: $P<0.05$; ***: $P<0.001$; n.s.: not significant; *versus* corresponding vehicle (veh)-treated groups

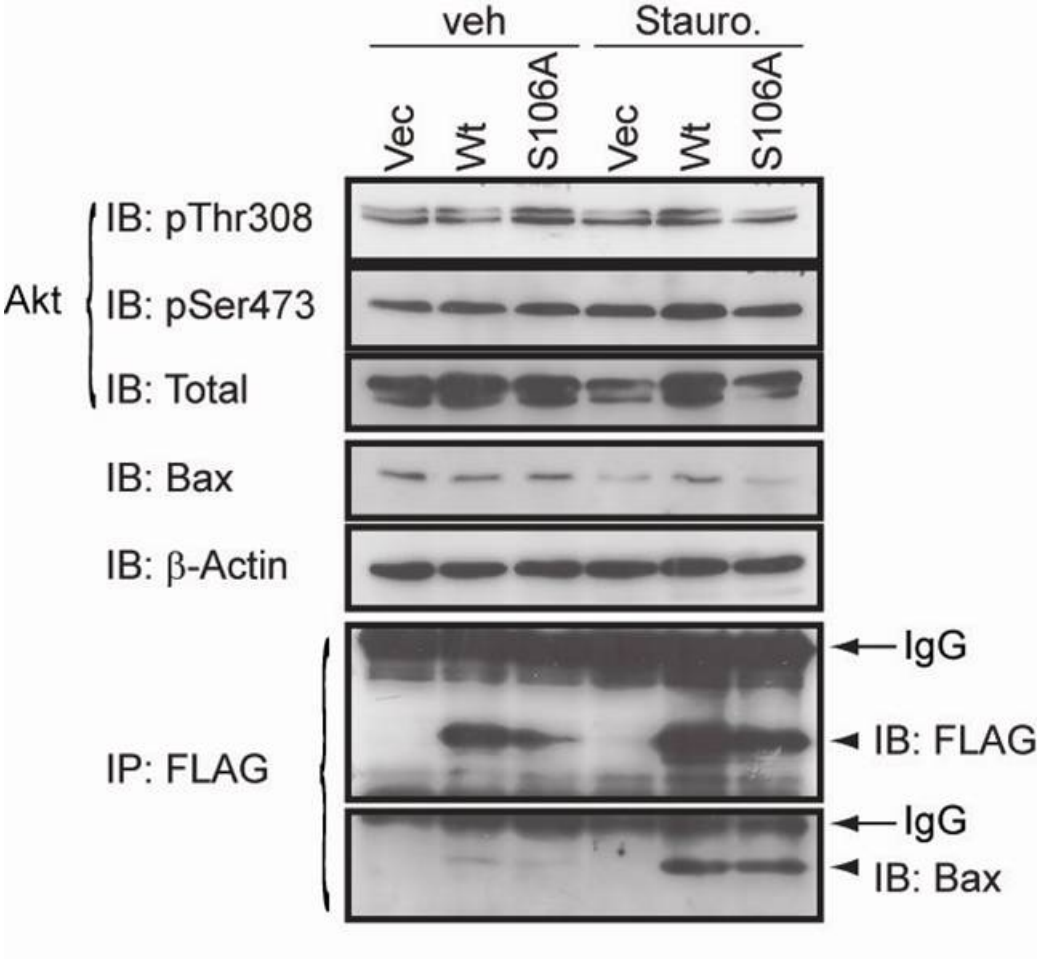


Figure 3.13: Bcl-xL(WT & S106A) interacts with pro-apoptotic Bax in staurosporine-treated PC12 cells. Staurosporine treatment diminishes total Akt expression as well as the expression of Bax (as it also does in primary culture, see Fig. 3.10). This is rescued by overexpression of Bcl-xL wildtype but not by the S106A mutant, although both Bcl-xL wildtype and S106A mutant associate with Bax in ST-treated PC12 cultures.

known interactor of Bcl-xL [91]). Both FLAG-Bcl-xL wildtype and S106A co-immunoprecipitated better with Bax upon treatment with ST. Interestingly, treatment with ST diminished both Akt and Bax expression (as had already been observed in primary culture, see Fig. 3.10). This was rescued by overexpression of Bcl-xL wildtype but not by the S106A mutant. Furthermore, Bcl-xL wildtype protected Akt expression (as well as the phosphorylation status of both Ser473 and Thr308) in the presence of ST. β-Actin expression revealed equal protein loading in all lanes (Fig. 3.13).

A co-immunoprecipitation experiment was performed to determine how inhibition of the PI3K/Akt pathway would affect the interaction between [FLAG] Bcl-xL and Bax. Bcl-xL WT and S106A were overexpressed in PC12 cells for 24 hours and treated for a further 24 h with 1 μM staurosporine in the absence or presence of 25 μM LY249002 (LY: a PI3K/Akt inhibitor). 48 hours post-transfection the cells were harvested and pre-cleared cell lysates were then immunoprecipitated for FLAG and for Akt. The SDS-PAGE resolved proteins revealed that FLAG-Bcl-xL(S106A) co-immunoprecipitated better with Akt than did the FLAG-Bcl-WT (Fig. 3.14). This interaction was further enhanced by treatment with LY, but was equally as strong for the WT and S106A variants. This same trend was observed for the interaction between Bcl-xL (WT and S106A) and Bax (Fig. 3.14). Akt was not dephosphorylated following treatment with LY. Although this is not what is expected of a PI3K/Akt inhibitor, it was not surprising as our laboratory had already noticed that LY inhibits PI3K/Akt quite readily in naïve cells, but that it is not as effective, and can actually activate Akt, in transfected cells [92]. The effect of LY was confirmed, however, by the loss of Akt-Ser473 phosphorylation in Bcl-xL(S106A)-expressing cells as well as by the increase in immunodetection of the caspase-3 (17kDa) cleavage product in the vector- and Bcl-xL

wildtype-transfected cells (Fig. 3.14). While the effect of the PI3K inhibitor, LY, in these cells was unexpected, these data suggest that substitution within the putative Akt consensus sequence does not hinder the interaction between Bcl-xL and Akt, but that the interaction between Bcl-xL and Akt could affect signalling events downstream of Akt.

Since Bcl-xL may be influenced by Akt, immunofluorescence staining was used to examine PC12 cells overexpressing Bcl-xL and treated with 1μM of staurosporine (ST) for 24 hours. The nuclei were stained with DAPI (Blue) and the overexpression of Bcl-xL was visualized using anti-FLAG antibody (green). This demonstrates that staurosporine induces PC12 cell neurite outgrowth (shown by green fluorescence) and that this effect is potentiated by overexpression of Bcl-xL(S106A) (Fig. 3.15). Different species of the differentiation-associated MAP-2 protein (microtubule-associated protein-2; [93]) were increased in PC12 cultures overexpressing Bcl-xL(S106A) and treated with ST (Fig. 3.16), thus corroborating the cellular differentiation observed in Fig. 3.15.

Finally, I chose to examine the potential for interaction of Bcl-xL(S106A) with VDAC, a component of the mitochondrial membrane pore complex and a known target for Bcl-xL in certain contexts [94]. The FLAG-tagged Bcl-xL wildtype and S106A variant were immunoprecipitated from precleared lysates. The FLAG-Bcl-xL WT and S106A proteins do not co-immunoprecipitate with VDAC (levels of VDAC remains the same level in total cell lysate) (Fig.3.17). This demonstrates that Bcl-xL(S106A) exerts its effects *via* a mechanism that is distinct from the mechanism of action of its pro-apoptotic analogue Bcl-xS(S106A).

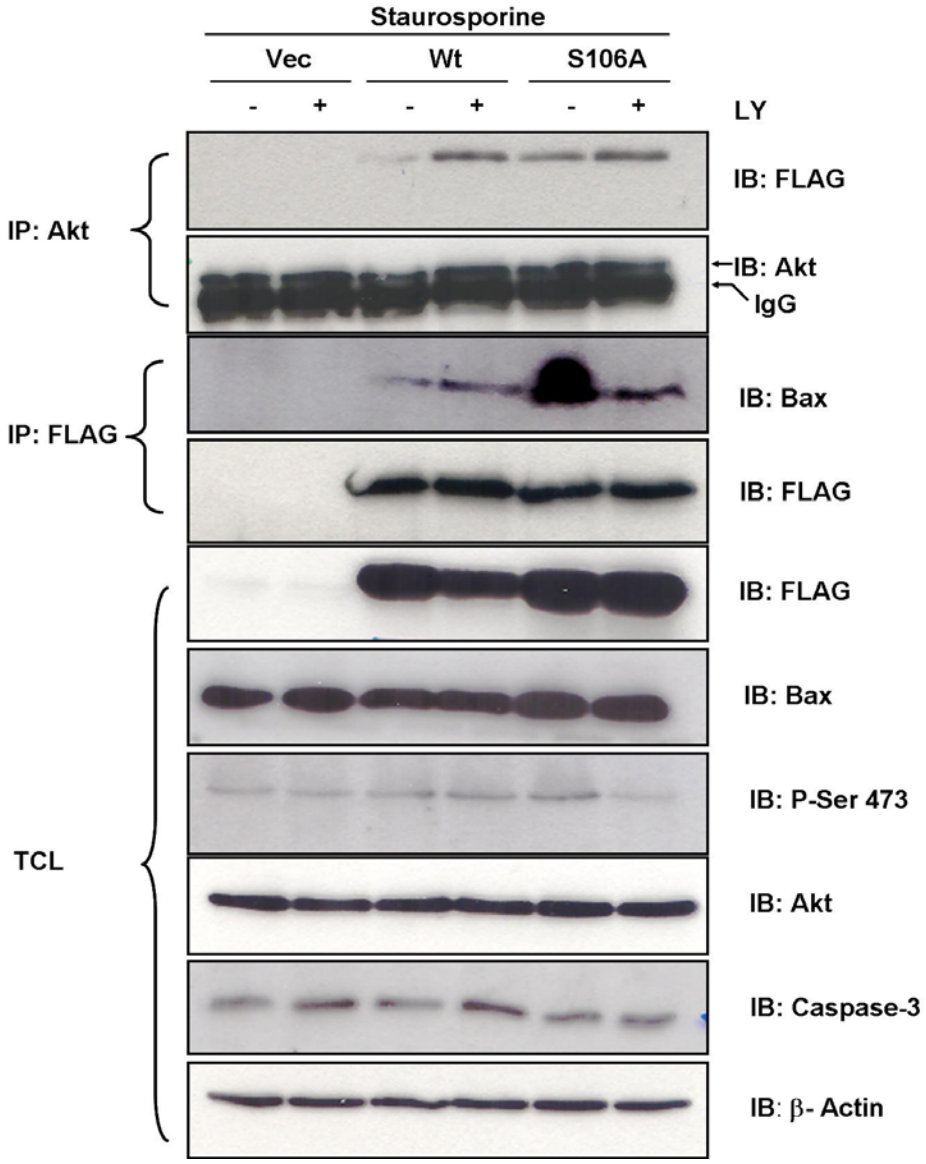
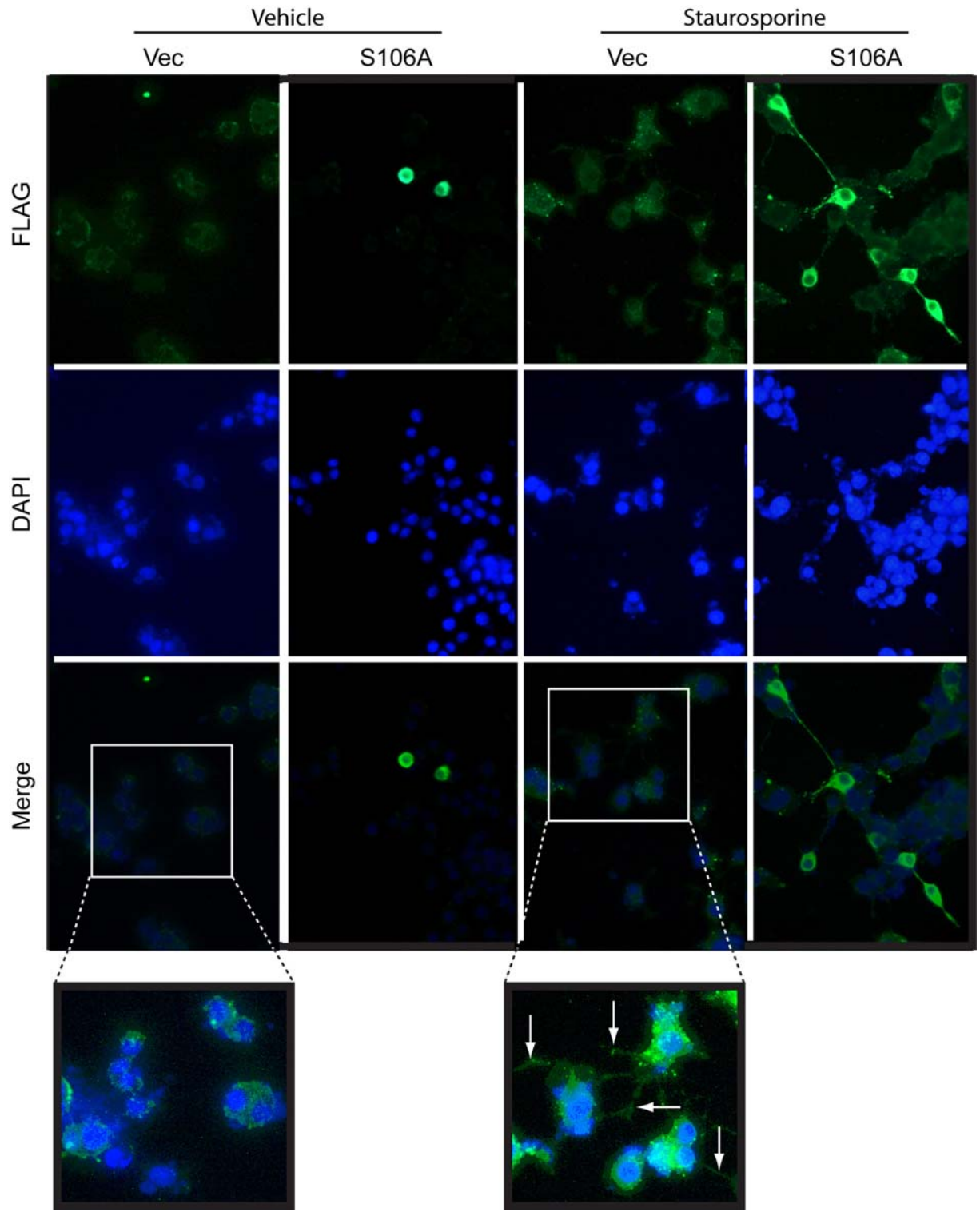


Figure 3.14: Mutation of Bcl-xL at Ser106 (a potential Akt target) induces more of an interaction with Akt in staurosporine-treated PC12 cells. Bcl-xL WT and mutation were overexpressed in PC12 cells for 48 hours, and co-treated with 1 μM staurosporine and 20 μM LY (PI3K inhibitor). The FLAG-Bcl-xL(S106A) variant co-immunoprecipitated better with the total-Akt protein than did the FLAG-Bcl-WT protein. IgG: heavy chain of the immunoprecipitating antibody.

Figure 3.15: (next page) Bcl-xL(S106A) promotes ST-induced differentiation of PC12 cells. PC12 cells expressing the GFP-Bcl-xL(S106A) variant (seen here in green) were treated with vehicle (veh) or staurosporine (ST) for 24 hours. Neurite outgrowth was used as a 'read-out' of differentiation. Nuclei were stained with DAPI (Blue). The intensity of the two images at the bottom was purposefully increased so as to visualize the very fine neurites in these cultures.


Vehicle
Staurosporine
Vec
S106A
Vec
S106A
FLAG
DAPI
Merge

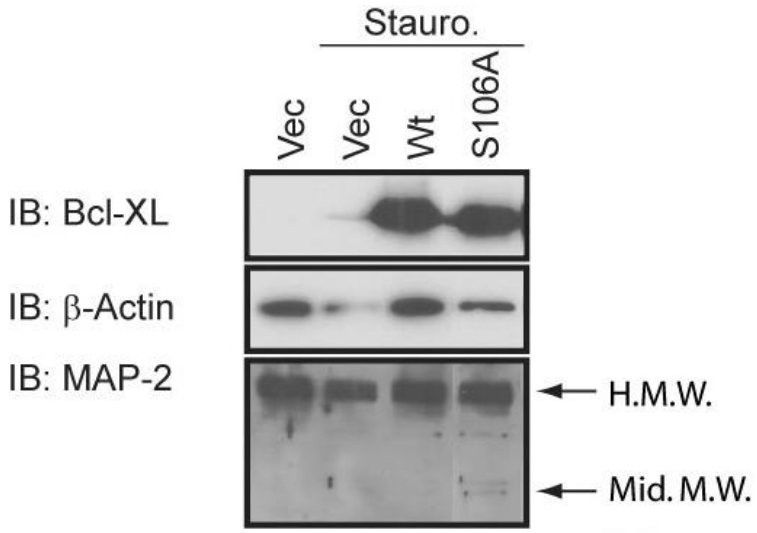


Figure 3.16: Staurosporine induces MAP-2 cleavage in Bcl-xL(S106A)-expressing PC12 cells. Bcl-xL WT and mutation were overexpressed in PC12 cells for 48 hours, and treated with 1μM staurosporine. The mutation of Bcl-xL in Ser106 residue has showed MAP-2 (neuronal specific protein) expression. H.M.W: high molecular weight (~250 kDa); mid.M.W: mid molecular weight (~65 kDa).

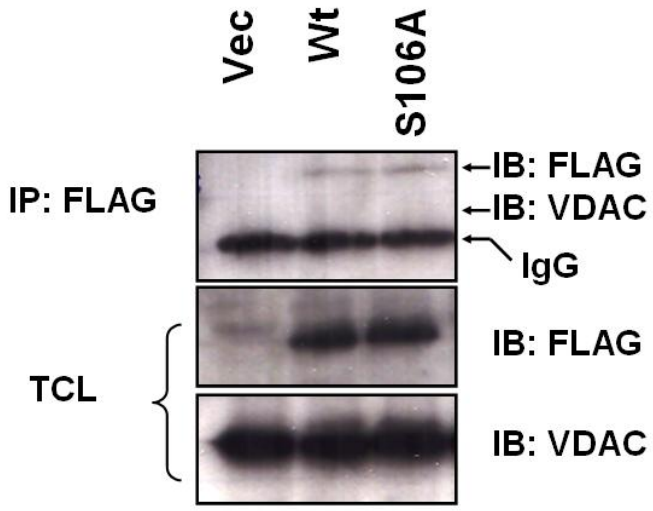


Figure 3.17: Neither Bcl-xL wildtype nor Bcl-xL(S106A) interacts with VDAC in PC12 cells. FLAG-tagged Bcl-xL wildtype (WT) and Bcl-xS(S106A) were overexpressed in PC12 cells. 24 hours post-transfection the cells were harvested and pre-cleared cell lysates were then immunoprecipitated with the FLAG antibody. Neither FLAG-Bcl-xL WT and FLAG-Bcl-xL(S106A) co-immunoprecipitated with VDAC. The overexpression of the FLAG-tagged proteins and the expression of endogenous VDAC were confirmed in total cell lysates (TCL). IgG: light chain of the immunoprecipitating antibody.

4. Discussion

The PI3K/Akt pathway exerts some of its effects on cell function *via* phosphorylation of effectors such as the Bcl-2 family of proteins. For example, Bcl-2-Thr56 can be phosphorylated during mitosis by CDK [32] and JNK [30]. While this might disable its anti-apoptotic function [30, 95], it would appear that multisite phosphorylation of Bcl-2 could stabilize and promote its anti-apoptotic function [33]. Multisite phosphorylation is also known to contribute to the function of Bad; Indeed, the phosphorylation of Bad-Ser112 by ERK and Ser136 by Akt [96, 97], on Ser155 (by PKA, but only if Ser136 is phosphorylated: [98]) and on Ser170 [42] all contribute to the tight control of this highly effective pro-apoptotic protein. Akt-mediated phosphorylation of Bax-Ser184 clearly impedes its translocation from the cytoplasm to mitochondrial targets [38]. JNK phosphorylates Bcl-XL within its activation loop on Ser62 [36] and possibly on threonine residues [37]. Such phosphorylation, particularly on Ser62, is known to contribute to the pro-apoptotic role for Bcl-xL as a consequence of diminished capacity to bind and disable pro-apoptotic Bax [99]. The phosphoregulation of pro-apoptotic Bcl-xS was, until recently, unclear, but now seems to also be regulated by multisite phosphorylation.

Part of this thesis work contributed to a recent publication [44] that was an extension of previous work from our laboratory. In it we had demonstrated that the antipsychotic haloperidol could induce Bcl-xS gene and protein expression, and that Bcl-xS could translocate to the mitochondria and facilitate cytochrome *c* release to the cytoplasm [87]. The mechanism that triggered the translocation of Bcl-xS was unclear, although a second study

clearly implicated a loss of Akt function as a major factor in haloperidol-mediated cytotoxicity [67]. With this in mind, our laboratory chose to examine the possibility that Bcl-xS was a candidate for phosphoregulation. My contribution was the demonstration that Bcl-xS proteins that bore directed S/A substitutions were toxic to PC12 cells. In particular, the toxicity of the S106A substitution within a putative Akt consensus motif contributed to cytochrome *c* release from the mitochondria and to a caspase-dependent loss of viability in these cells. The cytochrome *c* release was most probably a reflection of the increased ability of the Bcl-xS(S106A) protein to co-immunoprecipitate with the channel forming voltage-dependent anion channel (VDAC), a known factor in mitochondrial effects and cytochrome *c* release in other systems [94, 100, 101]. In support of the S106 residue being a novel target for Akt, it was observed that wildtype Bcl-xS co-immunoprecipitated with Akt, but that the S106A variant did not.

I observed that Bcl-xS(S106A)-induced cytotoxicity also relied on a mitochondrial event in HEK293A cells, but that there was no corresponding release of cytochrome *c*. Expression of Bcl-xS(S106) did, however, reduce the expression of the VDAC. While the loss of VDAC could explain the lack of any cytochrome *c* translocation to the cytoplasm, this did not explain why it was cytotoxic in these cells. It is possible, however, that the disruption of the VDAC-pore complex would effectively disrupt ATP flux and any ATP-dependent mechanisms, ultimately affecting cell function [102]. This is supported indirectly by the conspicuous absence in these cells of [caspase-dependent] PARP cleavage (an ATP-sensitive process in HEK cells; [103]). Others had previously observed that the mitochondria of Bcl-XS-expressing [non-neuronal] 3T3 fibroblasts were also almost transparent, indicating a lack of membrane content, and that this coincided with the cytosolic depletion of cytochrome *c* and

with caspase-independent death [104, 105].

At this point, I decided to investigate whether the anti-apoptotic Bcl-xL homologue was affected by phosphorylation on the same residues as we had already examined in the Bcl-xS studies, namely Bcl-xL Ser62, Ser106 and Ser228 (homologous to Ser165 in Bcl-xS, see Fig. 1.3). Although all three substitution variants expressed well in PC12 cells, only the S62A and the S106A variants induced the cleavage of caspase-9 and PARP, thus indicating an apoptotic phenotype. I again chose to focus on the S106A variant so as to compare and contrast the effects of an S106A substitution in both Bcl-xS and Bcl-xL. I also chose to use staurosporine, as it is a potent phospholipid/Ca^{2+}-dependent kinase inhibitor [73, 74] that induces caspase-dependent apoptotic cell death in neural stem cells [75] and osteoblasts [76], apparently by inhibiting Akt [77]. While I could have used haloperidol in these studies, I preferred to use staurosporine as it is used regularly in the published literature to study cytotoxicity *in vitro* [78-81], cell cycle arrest at G1/S phase [82], and also to study apoptosis and neurite outgrowth (a indicator of differentiation) in [pre]neuronal cells [83, 84, 106]. Treatment of PC12 cells with staurosporine decreased MTT conversion (an indicator of cell number/viability). The overexpression of Bcl-xL(S106A) tended to decrease MTT conversion on its own. While this effect was not significant, it did mitigate the 'significance' of the effect of staurosporine on MTT conversion. This, in combination with the observation that the effect of staurosporine alone or in combination with expressed Bcl-xL(S106A) affected the phosphorylation and expression of Akt, in addition to the fact that Bcl-xL(S106A) co-immunoprecipitated very well with pro-apoptotic Bax indicated two things. First, the effect of staurosporine could be mediated by a dephosphorylated Bcl-xL species and second, the combination of staurosporine and Bcl-xL(S106A) was not promoting an apoptotic phenotype

(because the interaction between Bcl-xL and Bax is a known protective mechanism: see ref. [35]). The latter conclusion was further confirmed by the inability of Bcl-xL(S106A) to interact with VDAC. The fact that Bcl-xL interacted with Akt (in staurosporine-treated PC12 cultures) and even more so with Bcl-xL(S106A), and that inhibition of PI3K with LY249002 enhanced the interaction between Bcl-xL wildtype, but not that of Bcl-xL(S106A), clearly implicated the PI3K/Akt cascade in the effects of Bcl-xL(S106A). Interestingly, the lack of a profound effect of LY249002 in transfected cells is not unexpected [92], but the loss of Akt phosphorylation in the Bcl-xL(S106A) cultures treated with staurosporine could be promoting a differentiating phenotype (see ref. [92]). In support of this, corresponding PC12 cell cultures clearly revealed morphological changes characteristic of differentiation. This was supported by the appearance of MAP-2 cleavage products that are usually associated with such differentiation phenotypes [93].

In summary, the Ser106 residues in both Bcl-xS and Bcl-xL appear to be targets for the Akt kinase. Interestingly, overexpression of the phospho-defective S106A variant can be either pro-apoptotic (in the case of Bcl-xS(S106A)) or pro-differentiation (in the case of Bcl-xL(S106A)). My work clearly demonstrates the contribution of Bcl-xS to the cytotoxicity profile of the antipsychotic haloperidol and also links the phosphoregulation of Bcl-xL to the effect of the kinase inhibitor staurosporine. Future studies would require an examination of the role of Bcl-xS/xL in the effector profile of both of these drugs given that haloperidol is also implicated in differentiation and that staurosporine is also involved in aspects of apoptosis. This is perhaps not surprising as differentiation requires a cell cycle arrest that, if held too long, leads to an apoptotic phenotype. Finally, determining the role of Bcl-xS/xL phosphoregulation in the profile of cancer therapeutics [44, 87, 107] would be interesting.

5. References

1. Hotchkiss RS and Nicholson DW (2006) Apoptosis and caspases regulate death and inflammation in sepsis. Nat Rev Immunol **6**: 813-822.
2. Gupta S (2001) Molecular steps of death receptor and mitochondrial pathways of apoptosis. Life Sci **69**: 2957-2964.
3. hStrasser A (2005) The role of BH3-only proteins in the immune system. Nat Rev Immunol **5**: 189-200.
4. Gupta S (2005) Molecular mechanisms of apoptosis in the cells of the immune system in human aging. Immunol Rev **205**: 114-129.
5. Gupta S (2002) A decision between life and death during TNF-alpha-induced signaling. J Clin Immunol **22**: 185-194.
6. Scaffidi C, Fulda S, Srinivasan A, et al. (1998) Two CD95 (APO-1/Fas) signaling pathways. Embo J **17**: 1675-1687.
7. Green DR and Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science **305**: 626-629.
8. Kharbanda S, Pandey P, Schofield L, et al. (1997) Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci U S A **94**: 6939-6942.
9. Chen ZQ, Jie X and Mo ZN (2008) [Curcumin inhibits growth, induces G1 arrest and apoptosis on human prostatic stromal cells by regulating Bcl-2/Bax]. Zhongguo Zhong Yao Za Zhi **33**: 2022-2025.
10. Simpson NH, Singh RP, Emery AN and Al-Rubeai M (1999) Bcl-2 over-expression reduces growth rate and prolongs G1 phase in continuous chemostat cultures of hybridoma cells. Biotechnol Bioeng **64**: 174-186.
11. Lutz RJ (2000) Role of the BH3 (Bcl-2 homology 3) domain in the regulation of apoptosis and Bcl-2-related proteins. Biochem Soc Trans **28**: 51-56.
12. Ruvolo PP, Deng X and May WS (2001) Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia **15**: 515-522.
13. Desagher S, Osen-Sand A, Montessuit S, et al. (2001) Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. Mol Cell **8**: 601-611.
14. Zinkel SS, Hurov KE, Ong C, et al. (2005) A role for proapoptotic BID in the DNA-damage response. Cell **122**: 579-591.
15. Kobayashi S, Lee SH, Meng XW, et al. (2007) Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1. J Biol Chem **282**: 18407-18417.
16. Domina AM, Vrana JA, Gregory MA, Hann SR and Craig RW (2004) MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. Oncogene **23**: 5301-5315.
17. Maurer U, Charvet C, Wagman AS, Dejardin E and Green DR (2006) Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell **21**: 749-760.

18. Inohara N, Gourley TS, Carrio R, et al. (1998) Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death. J Biol Chem **273**: 32479-32486.
19. Shimizu S, Ide T, Yanagida T and Tsujimoto Y (2000) Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome c. J Biol Chem **275**: 12321-12325.
20. Bartholomeusz G, Wu Y, Ali Seyed M, et al. (2006) Nuclear translocation of the pro-apoptotic Bcl-2 family member Bok induces apoptosis. Mol Carcinog **45**: 73-83.
21. Tan Y, Demeter MR, Ruan H and Comb MJ (2000) BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival. J Biol Chem **275**: 25865-25869.
22. Verma S, Zhao LJ and Chinnadurai G (2001) Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. J Biol Chem **276**: 4671-4676.
23. Willis SN and Adams JM (2005) Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol **17**: 617-625.
24. Coultas L, Terzano S, Thomas T, et al. (2007) Hrk/DP5 contributes to the apoptosis of select neuronal populations but is dispensable for haematopoietic cell apoptosis. J Cell Sci **120**: 2044-2052.
25. Puthalakath H, Huang DC, O'Reilly LA, King SM and Strasser A (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell **3**: 287-296.
26. Qi XJ, Wildey GM and Howe PH (2006) Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function. J Biol Chem **281**: 813-823.
27. Pezzella F, Turley H, Kuzu I, et al. (1993) bcl-2 protein in non-small-cell lung carcinoma. N Engl J Med **329**: 690-694.
28. Joensuu H, Pylkkanen L and Toikkanen S (1994) Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol **145**: 1191-1198.
29. Kutuk O and Letai A (2008) Regulation of Bcl-2 family proteins by posttranslational modifications. Curr Mol Med **8**: 102-118.
30. Yamamoto K, Ichijo H and Korsmeyer SJ (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol **19**: 8469-8478.
31. Ito T, Deng X, Carr B and May WS (1997) Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem **272**: 11671-11673.
32. Du L, Lyle CS, Obey TB, et al. (2004) Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: evidence that mitotic Bcl-2 phosphorylation is JNK-independent. J Biol Chem **279**: 11957-11966.
33. Brichese L, Barboule N, Heliez C and Valette A (2002) Bcl-2 phosphorylation and proteasome-dependent degradation induced by paclitaxel treatment: consequences on sensitivity of isolated mitochondria to Bid. Exp Cell Res **278**: 101-111.
34. Pattingre S, Bauvy C, Carpentier S, et al. (2009) Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. J Biol Chem **284**: 2719-2728.
35. Upreti M, Galitovskaya EN, Chu R, et al. (2008) Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance. J Biol Chem **283**: 35517-35525.

36. Basu A and Haldar S (2003) Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. FEBS Lett **538**: 41-47.
37. Kharbanda S, Saxena S, Yoshida K, et al. (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. J Biol Chem **275**: 322-327.
38. Gardai SJ, Hildeman DA, Frankel SK, et al. (2004) Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. J Biol Chem **279**: 21085-21095.
39. Linseman DA, Butts BD, Precht TA, et al. (2004) Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J Neurosci **24**: 9993-10002.
40. del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science **278**: 687-689.
41. Virdee K, Parone PA and Tolkovsky AM (2000) Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. Curr Biol **10**: 1151-1154.
42. Dramsi S, Scheid MP, Maiti A, et al. (2002) Identification of a novel phosphorylation site, Ser-170, as a regulator of bad pro-apoptotic activity. J Biol Chem **277**: 6399-6405.
43. Lindenboim L, Yuan J and Stein R (2000) Bcl-xS and Bax induce different apoptotic pathways in PC12 cells. Oncogene **19**: 1783-1793.
44. Wei Z, Qi J, Dai Y, Bowen WD and Mousseau DD (2009) Haloperidol disrupts Akt signalling to reveal a phosphorylation-dependent regulation of pro-apoptotic Bcl-XS function. Cell Signal **21**: 161-168.
45. Naumann U, Wischhusen J, Weit S, et al. (2004) Alkylphosphocholine-induced glioma cell death is BCL-X(L)-sensitive, caspase-independent and characterized by massive cytoplasmic vacuole formation. Cell Death Differ **11**: 1326-1341.
46. Shimizu S, Konishi A, Kodama T and Tsujimoto Y (2000) BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc Natl Acad Sci U S A **97**: 3100-3105.
47. Zamzami N and Kroemer G (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol **2**: 67-71.
48. Abou-Sleiman PM, Muqit MM and Wood NW (2006) Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat Rev Neurosci **7**: 207-219.
49. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275**: 1132-1136.
50. Rostovtseva TK, Antonsson B, Suzuki M, et al. (2004) Bid, but not Bax, regulates VDAC channels. J Biol Chem **279**: 13575-13583.
51. Shimizu S, Narita M and Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399**: 483-487.
52. Kim D, Cheng GZ, Lindsley CW, Yang H and Cheng JQ (2005) Targeting the phosphatidylinositol-3 kinase/Akt pathway for the treatment of cancer. Curr Opin Investig Drugs **6**: 1250-1258.

53. Kim R (2005) Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem Biophys Res Commun **333**: 336-343.
54. Carnero A, Blanco-Aparicio C, Renner O, Link W and Leal JF (2008) The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. Curr Cancer Drug Targets **8**: 187-198.
55. Chang F, Lee JT, Navolanic PM, et al. (2003) Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia **17**: 590-603.
56. Cheng GZ, Park S, Shu S, et al. (2008) Advances of AKT pathway in human oncogenesis and as a target for anti-cancer drug discovery. Curr Cancer Drug Targets **8**: 2-6.
57. Jiang BH and Liu LZ (2008) PI3K/PTEN signaling in tumorigenesis and angiogenesis. Biochim Biophys Acta **1784**: 150-158.
58. Blanco-Aparicio C, Renner O, Leal JF and Carnero A (2007) PTEN, more than the AKT pathway. Carcinogenesis **28**: 1379-1386.
59. Datta SR, Brunet A and Greenberg ME (1999) Cellular survival: a play in three Akts. Genes Dev **13**: 2905-2927.
60. Pap M and Cooper GM (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem **273**: 19929-19932.
61. Alessi DR, Kozlowski MT, Weng QP, Morrice N and Avruch J (1998) 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Curr Biol **8**: 69-81.
62. Mora A, Komander D, van Aalten DM and Alessi DR (2004) PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol **15**: 161-170.
63. Datta SR, Dudek H, Tao X, et al. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91**: 231-241.
64. Kandel ES and Hay N (1999) The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res **253**: 210-229.
65. Reddy KK, Lefkove B, Chen LB, et al. (2008) The antidepressant sertraline downregulates Akt and has activity against melanoma cells. Pigment Cell Melanoma Res **21**: 451-456.
66. Choi JH, Yang YR, Lee SK, et al. (2008) Potential inhibition of PDK1/Akt signaling by phenothiazines suppresses cancer cell proliferation and survival. Ann N Y Acad Sci **1138**: 393-403.
67. Dai Y, Wei Z, Sephton CF, et al. (2007) Haloperidol induces the nuclear translocation of phosphatidylinositol 3'-kinase to disrupt Akt phosphorylation in PC12 cells. J Psychiatry Neurosci **32**: 323-330.
68. Joy CB, Adams CE and Lawrie SM (2006) Haloperidol versus placebo for schizophrenia. Cochrane Database Syst Rev CD003082.
69. Pillai A, Veeranan-Karmegam R, Dhandapani KM and Mahadik SP (2008) Cystamine prevents haloperidol-induced decrease of BDNF/TrkB signaling in mouse frontal cortex. J Neurochem **107**: 941-951.
70. Rybczynska AA, Dierckx RA, Ishiwata K, Elsinga PH and van Waarde A (2008) Cytotoxicity of sigma-receptor ligands is associated with major changes of cellular

metabolism and complete occupancy of the sigma-2 subpopulation. J Nucl Med **49**: 2049-2056.
71. Terry AV, Jr., Parikh V, Gearhart DA, et al. (2006) Time-dependent effects of haloperidol and ziprasidone on nerve growth factor, cholinergic neurons, and spatial learning in rats. J Pharmacol Exp Ther **318**: 709-724.
72. Pillai A, Dhandapani KM, Pillai BA, Terry AV, Jr. and Mahadik SP (2008) Erythropoietin prevents haloperidol treatment-induced neuronal apoptosis through regulation of BDNF. Neuropsychopharmacology **33**: 1942-1951.
73. Budworth J and Gescher A (1995) Differential inhibition of cytosolic and membrane-derived protein kinase C activity by staurosporine and other kinase inhibitors. FEBS Lett **362**: 139-142.
74. Tamaoki T, Nomoto H, Takahashi I, et al. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. Biochem Biophys Res Commun **135**: 397-402.
75. Ceccatelli S, Tamm C, Sleeper E and Orrenius S (2004) Neural stem cells and cell death. Toxicol Lett **149**: 59-66.
76. Chae HJ, Kang JS, Byun JO, et al. (2000) Molecular mechanism of staurosporine-induced apoptosis in osteoblasts. Pharmacol Res **42**: 373-381.
77. Hill MM, Andjelkovic M, Brazil DP, et al. (2001) Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. J Biol Chem **276**: 25643-25646.
78. Mikami M, Goubaeva F, Song JH, Lee HT and Yang J (2008) beta-Adrenoceptor blockers protect against staurosporine-induced apoptosis in SH-SY5Y neuroblastoma cells. Eur J Pharmacol **589**: 14-21.
79. Numata T, Sato K, Okada Y and Wehner F (2008) Hypertonicity-induced cation channels rescue cells from staurosporine-elicited apoptosis. Apoptosis **13**: 895-903.
80. Wang S, Wang JA, Li J, Zhou J and Wang H (2008) Voltage-dependent potassium channels are involved in staurosporine-induced apoptosis of rat mesenchymal stem cells. Cell Biol Int **32**: 312-319.
81. Wang Y, Zhang B, Peng X, Perpetua M and Harbrecht BG (2008) Bcl-xL prevents staurosporine-induced hepatocyte apoptosis by restoring protein kinase B/mitogen-activated protein kinase activity and mitochondria integrity. J Cell Physiol **215**: 676-683.
82. Senderowicz AM (2003) Cell cycle modulators for the treatment of lung malignancies. Clin Lung Cancer **5**: 158-168.
83. Rasouly D, Rahamim E, Ringel I, et al. (1994) Neurites induced by staurosporine in PC12 cells are resistant to colchicine and express high levels of tau proteins. Mol Pharmacol **45**: 29-35.
84. Reuter H, Bouron A, Neuhaus R, Becker C and Reber BF (1992) Inhibition of protein kinases in rat pheochromocytoma (PC12) cells promotes morphological differentiation and down-regulates ion channel expression. Proc Biol Sci **249**: 211-216.
85. Adler EM, Gough NR and Blundon JA (2006) Differentiation of PC12 cells. Sci STKE **2006**: tr9.
86. Greene LA and Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci U S A **73**: 2424-2428.

87. Wei Z, Mousseau DD, Dai Y, Cao X and Li XM (2006) Haloperidol induces apoptosis via the sigma2 receptor system and Bcl-XS. Pharmacogenomics J **6**: 279-288.
88. Thakran P, Leuschen MP and Ebadi M (1989) Metallothionein induction in rat hippocampal neurons in primary culture. In Vivo **3**: 191-197.
89. Nechushtan A, Smith CL, Hsu YT and Youle RJ (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. Embo J **18**: 2330-2341.
90. Yu SW, Wang H, Poitras MF, et al. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science **297**: 259-263.
91. Billen LP, Kokoski CL, Lovell JF, Leber B and Andrews DW (2008) Bcl-XL inhibits membrane permeabilization by competing with Bax. PLoS Biol **6**: e147.
92. Sephton CF, Zhang D, Lehmann TM, et al. (2009) The nuclear localization of 3'-phosphoinositide-dependent kinase-1 is dependent on its association with the protein tyrosine phosphatase SHP-1. Cell Signal **21**: 1634-1644.
93. Tucker RP, Binder LI, Viereck C, Hemmings BA and Matus AI (1988) The sequential appearance of low- and high-molecular-weight forms of MAP2 in the developing cerebellum. J Neurosci **8**: 4503-4512.
94. Vander Heiden MG, Li XX, Gottleib E, et al. (2001) Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. J Biol Chem **276**: 19414-19419.
95. Srivastava RK, Mi QS, Hardwick JM and Longo DL (1999) Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. Proc Natl Acad Sci U S A **96**: 3775-3780.
96. Scheid MP, Schubert KM and Duronio V (1999) Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. J Biol Chem **274**: 31108-31113.
97. Hayakawa J, Ohmichi M, Kurachi H, et al. (2000) Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin. Cancer Res **60**: 5988-5994.
98. Datta SR, Katsov A, Hu L, et al. (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell **6**: 41-51.
99. Upreti M, Chu R, Galitovskaya E, Smart SK and Chambers TC (2008) Key role for Bak activation and Bak-Bax interaction in the apoptotic response to vinblastine. Mol Cancer Ther **7**: 2224-2232.
100. Martinou JC, Desagher S and Antonsson B (2000) Cytochrome c release from mitochondria: all or nothing. Nat Cell Biol **2**: E41-43.
101. Shi Y, Chen J, Weng C, et al. (2003) Identification of the protein-protein contact site and interaction mode of human VDAC1 with Bcl-2 family proteins. Biochem Biophys Res Commun **305**: 989-996.
102. Temkin V, Huang Q, Liu H, Osada H and Pope RM (2006) Inhibition of ADP/ATP exchange in receptor-interacting protein-mediated necrosis. Mol Cell Biol **26**: 2215-2225.
103. Wen LT, Caldwell CC and Knowles AF (2003) Poly(ADP-ribose) polymerase activation and changes in Bax protein expression associated with extracellular ATP-

mediated apoptosis in human embryonic kidney 293-P2X7 cells. Mol Pharmacol **63**: 706-713.
104. Fridman JS, Parsels J, Rehemtulla A and Maybaum J (2001) Cytochrome c depletion upon expression of Bcl-XS. J Biol Chem **276**: 4205-4210.
105. Fridman JS, Benedict MA and Maybaum J (1999) bcl-X(S)-induced cell death in 3T3 cells does not require or induce caspase activation. Cancer Res **59**: 5999-6004.
106. Yuste VJ, Sanchez-Lopez I, Sole C, et al. (2002) The prevention of the staurosporine-induced apoptosis by Bcl-X(L), but not by Bcl-2 or caspase inhibitors, allows the extensive differentiation of human neuroblastoma cells. J Neurochem **80**: 126-139.
107. Kfir S, Sionov RV, Zafrir E, Zilberman Y and Yefenof E (2007) Staurosporine sensitizes T lymphoma cells to glucocorticoid-induced apoptosis: role of Nur77 and Bcl-2. Cell Cycle **6**: 3086-3096.

6. Appendix

License agreement/permission for use of figures

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Jan 10, 2010

This is a License Agreement between JI QI ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2167191044215
License date	Apr 13, 2009
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	The role of BH3-only proteins in the immune system
Licensed content author	Andreas Strasser
Volume number	
Issue number	
Pages	
Year of publication	2005
Portion used	Figures / tables
Number of figures / tables	1
Requestor type	Student
Type of Use	Thesis / Dissertation
Billing Type	Invoice
Company	JI QI
Billing Address	B46 health sciences building 107 wiggins road Saskatoon, SK S7N 5E5 Canada
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you

have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 30 days of the license date. Payment should be in the form of a check or money order referencing your account number and this license number 2167191044215.
If you would prefer to pay for this license by credit card, please go to http://www.copyright.com/creditcard to download our credit card payment authorization form.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2167191044215 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Jan 10, 2010

This is a License Agreement between JI QI ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2167191366090
License date	Apr 13, 2009
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Apoptosis and caspases regulate death and inflammation in sepsis
Licensed content author	Richard S. Hotchkiss and Donald W. Nicholson
Volume number	6
Issue number	11
Pages	pp813-822
Year of publication	2006
Portion used	Figures / tables
Number of figures / tables	1
Requestor type	Student
Type of Use	Thesis / Dissertation
Billing Type	Invoice
Company	JI QI
Billing Address	B46 health sciences building 107 wiggins road Saskatoon, SK S7N 5E5 Canada
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you

have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 30 days of the license date. Payment should be in the form of a check or money order referencing your account number and this license number 2167191366090.
If you would prefer to pay for this license by credit card, please go to http://www.copyright.com/creditcard to download our credit card payment authorization form.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2167191366090 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 10, 2010

This is a License Agreement between JI QI ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	JI QI
Customer address	B46 health sciences building Saskatoon, SK S7N 5E5
License Number	2175561129859
License date	Apr 24, 2009
Licensed content publisher	Elsevier
Licensed content publication	Cellular Signalling
Licensed content title	Haloperidol disrupts Akt signalling to reveal a phosphorylation-dependent regulation of pro-apoptotic Bcl-XS function
Licensed content author	Zelan Wei, Ji Qi, Yunxiu Dai, Wayne D. Bowen and Darrell D. Mousseau
Licensed content date	January 2009
Volume number	
Issue number	
Pages	0
Type of Use	Thesis / Dissertation
Portion	Figures/table/illustration/abstracts
Portion Quantity	1
Format	Electronic
You are an author of the Elsevier article	Yes
Are you translating?	No
Order Reference Number	
Expected publication date	Aug 2009
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD

Total 0.00 USD

Terms and Conditions

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 30 days of the license date. Payment should be in the form of a check or money order referencing your account number and this license number 2175561129859.
If you would prefer to pay for this license by credit card, please go to http://www.copyright.com/creditcard to download our credit card payment authorization form.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2175561129859 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 10, 2010

This is a License Agreement between JI QI ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	JI QI
Customer address	B46 health sciences building Saskatoon, SK S7N 5E5
License Number	2220900666957
License date	Jul 02, 2009
Licensed content publisher	Elsevier
Licensed content publication	Cellular Signalling
Licensed content title	Haloperidol disrupts Akt signalling to reveal a phosphorylation-dependent regulation of pro-apoptotic Bcl-XS function
Licensed content author	Zelan Wei, Ji Qi, Yunxiu Dai, Wayne D. Bowen and Darrell D. Mousseau
Licensed content date	January 2009
Volume number	
Issue number	
Pages	0
Type of Use	Thesis / Dissertation
Portion	Figures/table/illustration/abstracts
Portion Quantity	3
Format	Electronic
You are an author of the Elsevier article	Yes
Are you translating?	No
Order Reference Number	
Expected publication date	Aug 2009
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.00 USD

Total 0.00 USD

Terms and Conditions

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 30 days of the license date. Payment should be in the form of a check or money order referencing your account number and this license number 2220900666957.
If you would prefer to pay for this license by credit card, please go to http://www.copyright.com/creditcard to download our credit card payment authorization form.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2220900666957 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.